

**MAMMALIAN GENES INVOLVED IN RAPAMYCIN RESISTANCE AND  
TUMORGENESIS: RAPR6 GENES**

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This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 60/404,315, filed on August 15, 2002, which is incorporated by  
10 reference herein in its entirety.

**1. FIELD OF THE INVENTION**

The invention relates to a novel mammalian gene, termed the RapR6 gene, which is involved in rapamycin resistance and tumorigenesis, and to RapR6 gene products and  
15 derivatives and analogs thereof. The invention relates to fragments (and derivatives and analogs thereof) of a mammalian RapR6 gene product. The invention also provides methods of production of mammalian RapR6 gene products, and derivatives and analogs thereof. The present invention also relates to methods and compositions for regulating rapamycin resistance and tumorigenesis by modulating the expression of and/or activity of a  
20 RapR6 gene. The compositions of the invention include but are not limited to nucleic acid encoding RapR6 gene products and homologues, analogues, and deletions thereof, as well as antisense, ribozyme, triple helix, double-stranded RNA. Also provided are antibody and polypeptide molecules and small organic or inorganic molecules which bind to a RapR6 protein. The invention also relates to methods and compositions for treatment of diseases,  
25 e.g., cancers, by modulating the expression and/or activity of RapR6 gene alone or in conjunction with a rapamycin therapy. The invention also relates to methods and compositions for diagnosing and screening RapR6-mediated rapamycin resistance and/or tumorigenesis in patients. The invention further relates to host cells in which expression of a truncated fragment of a RapR6 protein can be reversibly switched on and off, and to  
30 methods of using RapR6 gene in evaluation and screening for drugs which regulate rapamycin resistance and/or tumorigenesis. The invention also relates to methods for generating genetically modified cells having altered sensitivity to rapamycin by knocking out a gene which mediates rapamycin resistance.

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**2. BACKGROUND OF THE INVENTION**

Rapamycin (also called sirolimus) is a lipophilic macrolide which was isolated in 1975 as a fungicide from a strain of *Streptomyces hygroscopicus* found in a soil sample on Easter Island (see, e.g., Huang et al., 2001, Cancer and Metastasis Rev. 20:69-78; Sehgal et

al., 1994, Medicinal Research Review 14:1-22). Total synthesis of rapamycin has been  
5 reported (see, e.g., Nicolaou et al., 1993, J. Am. Chem. Soc. 115:4419; Hayward et al.,  
1993, J. Am. Chem. Soc. 115: 9345). Rapamycin, or 9,10,12,13,14,21,22,23,24,25,26,27,  
32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-me-  
thylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4  
]oxaazacyclohentricontine-1,5,11,28,29(4H,6H,31H)-pentone, comprises a 31-membered  
10 ring including a pipercolinyl group and pyranose ring, a conjugated triene system and a  
tri-carbonyl region. It has 15 chiral centers, and thus a large number of possible  
stereoisomers. Rapamycin targets the protein mTOR (the mammalian target of rapamycin,  
a homolog of TOR1 and TOR2, targets of rapamycin 1 and 2 in yeast), a serine/threonine  
kinase belonging to the phosphatidylinositol 3-kinase (PI3K) family of kinases (see, e.g.,  
15 Huang et al., 2001, Cancer and Metastasis Rev. 20:69-78; Yu et al., 2001, Endocrine-  
Related Cancer 8:249-258; and Sabers et al., 1995, J. Biol. Chem. 270:815-822). mTOR  
has been identified as a central integrator of extra- and intracellular signals that initiate  
translation and transcription required for cell growth and proliferation (see, e.g., Huang et  
al., 2001, Cancer and Metastasis Rev. 20:69-78). In its action, rapamycin first binds to an  
20 intracellular receptor called FKBP-12 ("FK506 Binding Protein 12"). The rapamycin-  
FKBP-12 complex inhibits mTOR and therefore one or more of its downstream pathways,  
e.g., 4E-BP1 and p70S6K, to cause G1 cell cycle arrest. Derivatives of rapamycin, e.g., cell  
cycle inhibitor-779 (CCI-779), which is a rapamycin ester, are also reported to have such  
effect (see, e.g., Huang et al., 2001, Cancer and Metastasis Rev. 20:69-78; Yu et al., 2001,  
25 Endocrine-Related Cancer 8:249-258).

Rapamycin has been approved by the FDA as an immunosuppressant for prevention  
and treatment of graft rejection in organ transplant recipients and is currently marketed  
under the trade name "Rapamune®" by Wyeth. As an immunosuppressant, Rapamycin  
30 demonstrates a different mechanism of action as compared to traditional  
immunosuppressants in that rapamycin blocks the immune response by inhibiting the  
function of mTOR, thereby causing programmed cell death, or apoptosis, in T cells. Other  
commonly used immunosuppressants, such as cyclosporin and FK-506, work differently by  
binding to calcineurin, thereby blocking the  $\text{Ca}^{2+}$ -dependent signaling pathway to the  
35 nucleus of the T cell. These latter immunosuppressants may have severe side effects  
because they also inhibit calcineurin activity in non-immune cells. In contrast, rapamycin  
selectively blocks the proliferation of T cells.

Rapamycin is also under clinical trial as a cancer chemotherapy drug due to its  
5 ability to cause cell cycle arrest in the G1 phase and to induce apoptosis (see, e.g., Huang et al., 2001, *Cancer and Metastasis Rev.* 20:69-78; Yu et al., 2001, *Endocrine-Related Cancer* 8:249-258; Mills et al., 2001, *Proc. Natl. Acad. Sci. USA* 98:10031-10033; Neshat et al., 2001, *Proc. Natl. Acad. Sci. USA* 98:10314-10319; and Podsypanina et al., 2001, *Proc. Natl. Acad. Sci. USA* 98:10320-10325). Rapamycin has been shown to be able to arrest the  
10 growth of a variety of malignant cells, including cells derived from rhabdomyosarcoma, neuroblastoma and glioblastoma, small cell lung cancer, osteosarcoma, pancreatic cancer, breast and prostate cancer, murine melanoma and leukemia, and B-cell lymphoma.

However, many cell lines have been found to exhibit resistance to the growth-  
15 inhibitory effect of rapamycin (see, e.g., Huang et al., 2001, *Cancer and Metastasis Rev.* 20:69-78). Resistance to rapamycin has been reported as a result of mutations in TOR or mTOR. In yeast, strains which have in TOR1 and/or TOR2 mutations that render the encoded proteins lacking the ability for rapamycin-FKBP-12 complex binding have been shown to be resistant to rapamycin completely (Heitman et al., 1991, *Science* 253:905-909).  
20 In mammals, a mutant of mTOR which exhibits reduced affinity for rapamycin-FKBP-12 complex has been reported to cause a high level of resistance to rapamycin (Chen et al., 1995, *Proc. Natl. Acad. Sci. USA* 92:4947-4951). Resistance to rapamycin has also been reported as a result of mutations in FKBP-12. For example, a resistant phenotype has been shown to be associated with a mutation in a mammalian homolog of FKBP-12 which leads  
25 to decreased binding of rapamycin. Mutations in downstream cellular constituents, e.g., p70S6K, that may confer rapamycin resistance have also been reported (see, e.g., Huang et al., 2001, *Cancer and Metastasis Rev.* 20:69-78).

In addition to stable rapamycin resistant phenotypes such as those resulting from genetic mutations, acquired rapamycin resistance in cell lines has also been reported (see,  
30 e.g., Huang et al., 2001, *Cancer and Metastasis Rev.* 20:69-78; Dilling et al., 2000, *Proceedings of 91<sup>st</sup> Annual Meeting of the AACR #5110*). Such rapamycin resistant cell lines were obtained by growing the cells in continuous and increasing concentrations of rapamycin. The cell lines can be reverted to rapamycin sensitive by growing the cells in the absence of rapamycin. Furthermore, genetic mutations in certain tumor suppressor genes,  
35 e.g., p53 and PTEN ("phosphatase and tensin homolog deleted on chromosome ten"), have been reported to result in rapamycin hypersensitivity (see, e.g., Huang et al., 2001, *Cancer*

and Metastasis Rev. 20:69-78; Huang et al., 2001, Cancer Research 61:3373-3381; Yu et  
5 al., 2001, Proceedings of 92<sup>nd</sup> Annual Meeting of the AACR #5110).

Considering the central role of mTOR in cell signaling and rapamycin as a potent  
anti-cancer drug candidate, additional cellular constituents and/or pathways, both upstream  
and downstream of mTOR, that confer rapamycin resistance may exist. Such cellular  
10 constituents and/or pathways may also play a role in tumorigenesis. However, such  
upstream or downstream constituents of the pathways are not yet known.

Citation of references hereinabove shall not be construed as an admission that such  
references are prior art to the present invention.

### 15 3. SUMMARY OF THE INVENTION

The invention provides nucleotide sequences of a novel mammalian gene, the  
RapR6 gene, which is involved in rapamycin resistance and tumorigenesis, and amino acid  
sequences of the encoded proteins, and derivatives and analogs thereof. In one  
20 embodiment, the invention provides a purified mammalian RapR6 protein. In a preferred  
embodiment, the invention provides a RapR6 protein which comprises the amino acid  
sequence substantially as set forth in SEQ ID NO:3 or 11. In another preferred  
embodiment, the invention provides a RapR6 protein which is encoded by a nucleic acid  
capable of hybridizing to a DNA having a sequence consisting of the coding region of  
SEQ ID NO:2 or 10.

25 The invention provides an isolated nucleic acid encoding a mammalian RapR6  
protein. In one embodiment, the invention provides a nucleic acid molecule comprising a  
nucleotide sequence as set forth in SEQ ID NO:2 or 10. In a preferred embodiment, the  
nucleic acid molecule of the invention is a DNA molecule. The invention also provides an  
30 isolated nucleic acid comprising a nucleotide sequence complementary to a nucleotide  
sequence encoding a mammalian RapR6 protein. The invention also provides an isolated  
nucleic acid comprising a nucleotide sequence that is hybridizable to a nucleotide sequence  
encoding a mammalian RapR6 protein.

35 The invention also provides derivatives and analogs of a protein encoded by a  
RapR6 gene. In one embodiment, the invention provides a purified derivative or analog of  
a protein which displays one or more functional activities of a mammalian RapR6 protein.

In a preferred embodiment, the invention provides a RapR6 derivative or analog which is  
5 capable of binding to an antibody directed against a mammalian RapR6 protein.

The invention also provides fragments of a protein encoded by a RapR6 gene, or a  
derivative or analog thereof. In one embodiment, the invention provides a purified  
fragment of a mammalian RapR6 protein. In a preferred embodiment, the invention  
10 provides a RapR6 fragment which comprises a WD40 domain of a mammalian RapR6  
protein. In a specific embodiment, the invention provides a fragment of a human RapR6  
protein which comprises amino acids 24-63, 66-105, 108-154, 203-244, or 247-287 of the  
human RapR6 protein. In another preferred embodiment, the invention provides a RapR6  
fragment which comprises a transmembrane domain of a mammalian RapR6 protein. In a  
15 specific embodiment, the invention provides a fragment of a human RapR6 protein which  
comprises amino acids 1-21 or 210-232 of the human RapR6 protein. In still another  
preferred embodiment, the invention provides a RapR6 fragment which does not comprise a  
WD40 domain or a transmembrane domain of a mammalian RapR6 protein. In a specific  
embodiment, the invention provides a fragment of a human RapR6 protein which  
20 comprises amino acids 155-202 of the human RapR6 protein. The invention also provides a  
molecule which comprises any of such fragment of a mammalian RapR6 protein.

The invention also provides a protein comprising an amino acid sequence that has at  
least 60% or at least 90% identity to a domain of a mammalian RapR6 protein, in which the  
percentage identity is determined over an amino acid sequence of identical size to the  
25 domain. The invention also provides a polypeptide comprising a fragment of a mammalian  
RapR6 protein consisting of at least 6 amino acids fused via a covalent bond to an amino  
acid sequence of a second peptide which is not comprised in a mammalian RapR6 protein.  
In a preferred embodiment of the invention, such fragment of the mammalian RapR6  
protein is a fragment capable of binding to an anti-RapR6 protein antibody. In another  
30 preferred embodiment, the fragment that is capable of binding to an anti-RapR6 protein  
antibody lacks one or more domains of the RapR6 protein.

The invention also provides an antibody which is capable of binding to a  
mammalian RapR6 protein. In a preferred embodiment, the antibody of the invention is a  
35 monoclonal antibody. In another preferred embodiment, the invention provides a molecule  
comprising an antibody fragment which is capable of binding to a RapR6 protein.

The invention also provides an isolated nucleic acid comprising a fragment of a mammalian RapR6 gene consisting of at least 8 nucleotides. In a preferred embodiment, the invention provides an isolated nucleic acid comprising a fragment of a mammalian RapR6 gene comprising any one of exons 1-6 of a mammalian RapR6 gene. In another preferred embodiment, the invention provides an isolated nucleic acid comprising a fragment of a mammalian RapR6 gene comprising an intron, or a fragment thereof, of a mammalian RapR6 gene. The invention also provides an isolated nucleic acid comprising a nucleotide sequence encoding a fragment of a mammalian RapR6 protein that displays one or more functional activities of the mammalian RapR6 protein. The invention further provides an isolated nucleic acid comprising a nucleotide sequence encoding any one of the fragments of a RapR6 protein as set forth in the invention.

The invention also provides a recombinant cell containing a nucleic acid comprising a nucleotide sequence encoding a fragment of a mammalian RapR6 protein that displays one or more functional activities of the mammalian RapR6 protein.

The invention also provides methods of production of proteins encoded by a RapR6 gene, and derivatives and analogs thereof. In a preferred embodiment, the invention provides a method of producing a mammalian RapR6 protein comprising: (a) growing a recombinant cell containing a nucleic acid encoding a fragment of a mammalian RapR6 protein that displays one or more functional activities of the mammalian RapR6 protein such that the encoded fragment of said mammalian RapR6 protein is expressed by the cell; and (b) recovering the expressed fragment of the mammalian RapR6 protein. The invention also provides the product of the method.

The invention also provides pharmaceutical composition comprising a therapeutically effective amount of a mammalian RapR6 protein or a fragment thereof and a pharmaceutically acceptable carrier. The fragment can be of any size having the desired activity, e.g., 5, 10, 20, 50, 100, or 200 amino acids. The invention also provides a pharmaceutical composition comprising a therapeutically effective amount of an antibody capable of binding to a mammalian RapR6 protein and a pharmaceutically acceptable carrier.

The present invention also provides methods for generating a genetically modified cell having altered sensitivity to rapamycin. In one embodiment, the method comprises introducing randomly into the genome of a host cell of a selected cell type of an organism a

DNA construct which comprises (i) a regulated promoter and (ii) a selection marker coding  
5 sequence under the control of the regulated promoter; hereinafter, this DNA construct may  
be referred to as a "knockout construct." The regulated promoter, when activated, initiates  
RNA transcription to produce an RNA and genetically modified cells exhibiting the desired  
phenotype are selected, e.g., if the host cell are rapamycin resistant, the selected cell is  
rapamycin sensitive, or if the host cell is rapamycin sensitive, the selected cell is rapamycin  
10 resistant. In a preferred embodiment, the method further comprises, prior to the step of  
introducing the knockout DNA construct, introducing into the genome of cells of the  
selected cell type a DNA construct encoding a transactivator, which comprises (i) a  
promoter and (ii) a nucleotide sequence encoding the transactivator under the control of the  
promoter and the genetically modified cell is generated by introducing the knockout DNA  
15 construct into a cell comprising a DNA construct encoding a transactivator which can  
activate the regulated promoter. In a preferred embodiment, the regulated promoter is a  
tetracycline regulated promoter and the transactivator activates the regulated promoter in  
the absence of tetracycline. In another preferred embodiment, the regulated promoter is a  
tetracycline regulated promoter and the transactivator activates the regulated promoter in  
20 the presence of tetracycline.

The knockout DNA construct may further comprise a rapid cloning element which  
comprises a replication origin sequence comprising sequences for initiation of replication  
and segregation, e.g., an Ori, and a bacterial selection marker, e.g., a chloramphenicol  
25 resistance gene. In one embodiment, the method of the invention further comprises  
activating the regulated promoter and identifying the genetically modified cell by a method  
comprising identifying a change in rapamycin resistance in the genetically modified cell. In  
another embodiment, the method further comprises cloning a fragment of genomic  
sequence by a method comprising: (a) obtaining a nucleotide sequence comprising the rapid  
30 cloning element and the fragment of genomic sequence; (b) circularizing the nucleotide  
sequence to generate a circular plasmid; and (c) transforming a suitable host cell using the  
circular plasmid. The sequence of the fragment of genomic sequence can be determined by  
a method comprising sequencing the circular plasmid. The location of the fragment of  
genomic sequence can be determined by a method comprising comparing the sequences  
35 with the genomic sequence of the selected cell type.

In the methods, the host cell can be but is not limited to a human host cell or a  
murine host cell, whereas the selected cell type can be a rapamycin resistant cell type or a

rapamycin sensitive cell type. In a preferred embodiment, the cell type is a murine N2a cell  
5 line. In another preferred embodiment, the knockout DNA construct is integrated at a  
RapR6 locus. The engineered cells having the desired rapamycin phenotype can be used to  
screen or identify compounds that regulate rapamycin resistance.

A particular embodiment of the invention relates to a method for treating a mammal,  
10 e.g., a human, having a cancer which is caused by defective regulation of a RapR6 gene  
and/or defective activity of a protein encoded by the RapR6 gene. This aspect of the  
invention is based, in part, on the applicant's discovery that RapR6 is a cellular constituent  
that regulates rapamycin resistance and tumorigenesis. In particular, the data presented  
herein show that expression of a truncated fragment of RapR6 confers resistance to  
15 rapamycin. Thus, therapeutic regimens which downregulate expression or activity of  
RapR6 can be used to potentiate the effects of rapamycin; in particular, the antitumor  
effects of rapamycin. In one embodiment, the method comprises administering to the  
mammal a therapeutically sufficient amount of an agent which regulates the expression of  
the RapR6 gene and/or activity of the protein encoded by the RapR6 gene. In another  
20 embodiment, the cancer is caused by a mutation in the RapR6 gene, and the method  
comprises administering an agent causes the expression of a normal version of the RapR6  
gene in cells of the cancer. In still another embodiment, the method comprises  
administering an agent which comprises a RapR6 protein or a therapeutically equivalent  
fragment thereof.

25 The invention also provides a method for treating a mammal having a cancer,  
comprising administering to the mammal undergoing a rapamycin therapy a therapeutically  
sufficient amount of an agent which regulates the expression of a RapR6 gene and/or  
activity of a protein encoded by the RapR6 gene such that rapamycin resistance is regulated.  
In a specific embodiment, the invention provides a method for treating a mammal having a  
30 cancer, comprising administering to the mammal i) a therapeutically sufficient amount of an  
agent which regulates the expression of a RapR6 gene and/or activity of a protein encoded  
by the RapR6 gene such that rapamycin resistance is regulated, and ii) a therapeutically  
sufficient amount of rapamycin or an analog or derivative of rapamycin. In another  
embodiment, the agent causes the expression of a normal version of the RapR6 gene in cells  
35 of the cancer. In still another embodiment, the agent comprises a RapR6 protein or a  
therapeutically equivalent fragment thereof.



The invention also provides methods for diagnosing in a mammal a cancer which is  
5 a result of defective regulation of a RapR6 gene or a predisposition to such a cancer. In one  
embodiment, the method comprises determining an expression level of the RapR6 gene in  
cells of the mammal, in which an expression level deviated from a predetermined threshold  
level indicates that the mammal has or is predisposed of the cancer. In a preferred  
embodiment, the expression level of the RapR6 gene is determined by a method comprising  
10 measuring the expression level of the RapR6 gene using one or more polynucleotide probes,  
each of which comprises a nucleotide sequence in the RapR6 gene. In one embodiment, the  
one or more polynucleotide probes comprise at least one polynucleotide probe comprising a  
nucleotide sequence within one of exons 1-6 of the RapR6 gene. In another embodiment,  
the one or more polynucleotide probes comprise at least one polynucleotide probe  
15 comprising a nucleotide sequence within an intron of the RapR6 gene. In particularly  
preferred embodiments of the invention, the methods is used to diagnose a cancer in a  
human. In one embodiment, the one or more polynucleotide probes comprise at least one  
polynucleotide probe comprising a nucleotide sequence comprised in the nucleotide  
sequence encoding a WD domain or a transmembrane domain in a RapR6 protein.  
20 Preferably, the one or more polynucleotide probes are polynucleotide probes on a  
microarray.

In another embodiment, the invention provides a method for diagnosing in a  
mammal a cancer which is a result of defective regulation of a RapR6 gene or a  
25 predisposition to such a cancer comprising determining a level of abundance of a protein  
encoded by the RapR6 gene in cells of the mammal, in which a level of abundance of the  
protein deviated from a predetermined threshold level indicates that the mammal has or is  
predisposed of the cancer. In still another embodiment, the invention provides a method for  
diagnosing the cancer comprising determining a level of activity of a protein encoded by the  
30 RapR6 gene in cells of the mammal, in which an activity level deviated from a  
predetermined threshold level indicates that the mammal has or is predisposed of the  
cancer. In particularly preferred embodiments of the invention, the methods is used to  
diagnose a cancer in a human. In one embodiment, the protein is a human RapR6 protein as  
depicted in SEQ ID NO:11. In other preferred embodiments of the invention, the methods  
35 is used to diagnose a cancer in a mouse. In one embodiment, the protein is a murine RapR6  
protein as depicted in SEQ ID NO:3.

The invention also provides methods for evaluating rapamycin resistance in a cell.

- 5 In one embodiment, the method comprises determining an expression level of a RapR6 gene in the cell, in which an expression level deviated from a predetermined threshold level indicates that the cell is rapamycin resistant. In a preferred embodiment, the expression level of the RapR6 gene is determined by a method comprising measuring the expression level of the RapR6 gene using one or more polynucleotide probes, each of which comprises
- 10 a nucleotide sequence in the RapR6 gene. In one embodiment, the one or more polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide sequence within one of exons 1-6 of the RapR6 gene. In another embodiment, the one or more polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide sequence within an intron of the RapR6 gene. In particularly preferred
- 15 embodiments of the invention, the methods is used to evaluate rapamycin resistance in a human cell. In one embodiment, the one or more polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide sequence comprised in the nucleotide sequence encoding a WD40 domain or a transmembrane domain in a RapR6 protein. Preferably, the one or more polynucleotide probes are polynucleotide probes on a
- 20 microarray.

- In another embodiment, the invention provides a method for evaluating rapamycin resistance in a cell comprising determining a level of abundance of a protein encoded by a RapR6 gene in the cell, in which a level of abundance of the protein deviated from a
- 25 predetermined threshold level indicates that the cell is rapamycin resistant. In still another embodiment, the invention provides a method for evaluating rapamycin resistance in a cell comprising determining a level of activity of a protein encoded by the RapR6 gene in cells of the mammal, in which an activity level deviated from a predetermined threshold level indicates that the cell is rapamycin resistant. In particularly preferred embodiments of the
- 30 invention, the methods is used to evaluating rapamycin resistance in a human cell. In one embodiment, the protein is a human RapR6 protein as depicted in SEQ ID NO:11. In other preferred embodiments of the invention, the methods is used to evaluating rapamycin resistance in a murine cell. In one embodiment, the protein is a murine RapR6 protein as depicted in SEQ ID NO:3.

- 35 The present invention also provides a method for regulating rapamycin resistance in a cell. In one embodiment, the method comprises contacting the cell with a sufficient amount of an agent which regulates the expression of a RapR6 gene and/or the activity of a

protein encoded by the RapR6 gene such that rapamycin resistance is regulated. The  
5 invention also provides methods for regulating rapamycin resistance in a mammal,  
comprising administering to the mammal a therapeutically sufficient amount of an agent  
which regulates the expression of a RapR6 gene and/or the activity of a protein encoded by  
the RapR6 gene such that rapamycin resistance is regulated. The invention further provides  
a method for regulating the growth of a cell, comprising contacting the cell with i) a  
10 sufficient amount of an agent which regulates the expression of a RapR6 gene and/or the  
activity of a protein encoded by the RapR6 gene such that rapamycin resistance is  
regulated; and ii) a sufficient amount of rapamycin or an analog or derivative of rapamycin.  
In another embodiment, the agent causes the expression of a normal version of the RapR6  
gene in the cell. In still another embodiment, the agent comprises a RapR6 protein or a  
15 therapeutically equivalent fragment thereof.

The invention also provides a method of identifying an agent that is capable of  
regulating rapamycin resistance via its capability of modulating the expression of a RapR6  
gene and/or the activity of a protein encoded by the RapR6 gene. In one embodiment, the  
20 method comprises comparing the inhibitory effects of rapamycin on cells expressing the  
RapR6 gene in the presence of the agent and in the absence of the agent, and identifying the  
agent as capable of regulating rapamycin resistance if there is a difference in the inhibitory  
effects of rapamycin. In a specific embodiment, the invention provides a method  
comprising: (a) contacting a first cell expressing the RapR6 gene with rapamycin in the  
25 presence of the agent and measuring a first growth inhibitory effect; (b) contacting a second  
cell expressing the RapR6 gene with rapamycin in the absence of the agent and measuring a  
second growth inhibitory effect; and (c) comparing the first and second inhibitory effects as  
measured in step (a) and (b), and identifying the agent as capable of regulating rapamycin  
resistance if there is a difference between the first and second inhibitory effects. In another  
30 embodiment, the agent causes the expression of a normal version of the RapR6 gene in the  
cell. In still another embodiment, the agent comprises a RapR6 protein or a therapeutically  
equivalent fragment thereof.

The invention further provides methods of producing an antibody that binds  
specifically to a RapR6 protein. In one embodiment, the method comprises raising the  
35 antibody against the RapR6 protein or a polypeptide comprising an fragment of the RapR6  
protein. In one embodiment, the protein is a human RapR6 protein as depicted in SEQ ID  
NO:11. In other preferred embodiments of the invention, the RapR6 protein used to

produce the antibody is a murine RapR6 protein, e.g., the murine RapR6 protein as depicted  
5 in SEQ ID NO:3.

The invention further provides an antibody that binds specifically to a RapR6  
protein or a fragment of the RapR6 protein such that binding of the antibody to the RapR6  
protein regulates rapamycin resistance. In one embodiment, the antibody binds specifically  
10 to a human RapR6 protein. In another embodiment, the antibody binds specifically to a  
human RapR6 protein. In still another embodiment, the antibody binds specifically to a  
murine RapR6 protein.

The invention further provides an agent that regulates the expression of a RapR6  
gene such that rapamycin resistance is regulated. In one embodiment, the agent comprises a  
15 molecule which regulates the expression of the RapR6 gene. In another preferred  
embodiment, the agent causes the expression of a normal version of the RapR6 gene in a  
cell.

The invention further provides a cell comprising at a RapR6 locus a knockout DNA  
20 construct which comprises (i) a regulated promoter and (ii) a selection marker coding  
sequence under the control of the regulated promoter. In a cell of the invention, activation  
of the regulated promoter initiates RNA transcription to produce an antisense RNA. In a  
preferred embodiment, the cell of the invention further comprises a DNA construct which  
comprises (i) a promoter and (ii) a nucleotide sequence encoding a transactivator which can  
25 activate the regulated promoter, in which the nucleotide sequence is under the control of the  
promoter. The cell of the invention can also comprise a rapid cloning element comprising a  
replication origin sequence comprising sequences for initiation of replication and  
segregation, e.g., an Ori, and a bacterial selection marker, e.g., a chloramphenicol resistance  
gene. Preferably, in the cell of the invention, the regulated promoter is a tetracycline  
30 regulated promoter, and the transactivator binds to the regulated promoter in the absence of  
tetracycline. The cell of the invention can be a rapamycin resistant cell type or a rapamycin  
sensitive cell type. The cell of the invention can also be a human or a murine cell. In a  
preferred embodiment, the cell is a murine N2a cell. In another preferred embodiment, the  
integration site is in the intron between exon 1 and 2 of the RapR6 locus. The invention  
35 further provides a kit for screening for agents which regulate rapamycin resistance and/or  
tumorigenesis, comprising in one or more containers (i) the cell of the invention; (ii)

5 tetracycline or a derivative or analog thereof; and (iii) rapamycin or a derivative or analog thereof.

The invention further provides DNA microarrays for diagnosing rapamycin resistance. The microarray of the invention comprises one or more polynucleotide probes, each of which comprises a nucleotide sequence in a RapR6 gene. In one embodiment, the one or more polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide sequence within one of exons 1-6 of a RapR6 gene. In another embodiment, the one or more polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide sequence within an intron of the RapR6 gene. In still another embodiment, the one or more polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide sequence comprised in the nucleotide sequence encoding a WD40 domain or a transmembrane domain in a human RapR6 protein.

The invention further provides a kit for diagnosis of rapamycin resistance, comprising in one or more containers one or more polynucleotide probes, wherein each of the polynucleotide probes comprises a nucleotide sequence in a RapR6 gene.

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#### 4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A depicts a nucleotide sequence (SEQ ID NO:1) flanking the 5' side of the RHKO insertion site. The sequence was obtained from RHKO clone RapR6. FIG. 1B depicts alignment of sequences obtained from three RHKO clones RapR61, RapR62, and RapR63.

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FIGS. 2A-2B depict the cDNA sequence of the murine RapR6 gene (SEQ ID NO:2) and the encoded amino acid sequence of the murine RapR6 protein (SEQ ID NO:3). FIGS. 2C depict the nucleotide sequences of the murine exons 1-6 (SEQ ID NO:4 through SEQ ID NO:9).

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FIGS. 3A-3B depict the cDNA sequences and the translated amino acid sequences and the regulatory sequences of human RapR6. FIGS. 3A-3B: the cRNA sequence (SEQ ID NO:10, FIG. 3A) and the amino acid sequence (SEQ ID NO:11, FIG. 3B) of human RapR6.

35

FIG. 4A depicts an alignment of the cDNA sequences of the human and murine RapR6 genes. FIG. 4B depicts an alignment of the human and murine RapR6 protein sequences. M: murine; H: human.

FIG. 5A depicts the genomic region of the murine RapR6 gene. FIG. 5B depicts the location of the RHKO insertion site in the murine RapR6 gene. The RHKO vector is inserted in the intron between exon 1 and exon 2 of the murine RapR6 gene.

FIG. 6A depicts the genomic region of the human RapR6 gene. FIG. 6B depicts identified WD domains in the human RapR6 gene. FIG. 6C depicts identified transmembrane domains in the human RapR6 gene.

FIG. 7 illustrates sensitivity of the N2a cell line to the growth inhibitory effect of rapamycin. The growth of N2a cells is completely inhibited when the cells are treated with rapamycin at a concentration of 1  $\mu$ M or greater.

FIGS. 8A-8B illustrate reversible inhibition by rapamycin in RapR6 clone (MTT proliferation assay). 8A: Solid bar, measurement when the knockout construct is expressed; shaded bar, measurement when the expression of the knockout construct is suppressed; and open bar, control. 8B: Calculated reversibility R according to Equation 1. FIG. 8C illustrates RapR6 colony after 12 days of infection with an RHKO gene search vector. FIGS. 8D-8E illustrate markers of G1 arrest in RapR6 clone. Colony 6 is the RapR6 clone of the present invention. Colonies 1, 2, and 8 are clones which do not exhibit rapamycin resistance.

FIGS. 9A-9B depict exemplary knockout or gene search constructs. FIG. 9C depicts the retroviral vector used to introduce the knockout construct. TRE: Tetracycline Response Element. FIG. 9D depicts a construct which expresses the tetracycline-controlled transactivator (tTA) of a Tet-off system.

FIG. 10 depicts exemplary SNPs in human RapR6 gene.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The invention provides nucleotide sequence of a novel mammalian gene, the RapR6 gene, which is involved in rapamycin resistance and tumorigenesis, and amino acid sequences of the encoded proteins, and derivatives and analogs thereof. The invention

provides derivatives and analogs of a protein encoded by the RapR6 gene. The invention  
5 provides fragments (and derivatives and analogs thereof) of a protein encoded by the RapR6  
gene. The invention also provides methods of production of proteins encoded by the  
RapR6 gene, and derivatives and analogs thereof.

The present invention provides methods for identifying cellular constituents, e.g.,  
10 genes and proteins, and/or pathways that are involved in rapamycin resistance and/or  
tumorigenesis. The invention also provides methods for generating genetically modified  
cells having altered sensitivity to rapamycin by knocking out a gene which mediates  
rapamycin resistance. The present invention also provides methods and compositions for  
regulating rapamycin resistance and/or tumorigenesis by modulating such cellular  
15 constituents and/or pathways. In specific embodiments of the invention, the present  
invention provides mammalian RapR6 genes and proteins and derivatives and analogs  
thereof, fragments (and derivatives and analogs thereof) of a mammalian RapR6 protein,  
and methods for production of mammalian RapR6 proteins (and derivatives and analogs  
thereof). The invention also provides methods and compositions for regulating rapamycin  
20 resistance and/or tumorigenesis in a cell or organism by targeting RapR6 gene and/or  
protein. In the methods of the invention, rapamycin resistance and/or tumorigenesis is  
regulated, e.g., inhibited, reduced or enhanced, by modulating the expression of RapR6  
gene and/or the activity, e.g., the interaction of RapR6 gene with other intra- or extra-  
cellular molecules. The compositions of the invention include but not limited to nucleic  
25 acid encoding RapR6 gene and homologues, analogues, and deletions thereof, as well as  
antisense, ribozyme, triple helix, double-stranded RNA, antibody and polypeptide  
molecules, and small organic or inorganic molecules. The invention also provides methods  
and compositions for treatment of diseases, e.g., cancers, by modulating the activity of  
RapR6 gene in conjunction with a rapamycin therapy. The invention also provides methods  
30 and compositions for diagnosing and screening RapR6 mediated rapamycin resistance  
and/or tumorigenesis in patients. The invention further provides host cells expressing a  
truncated fragment of a RapR6 protein reversibly, and methods of using RapR6 gene in  
evaluation and screening for drugs which modulate rapamycin resistance and/or  
tumorigenesis, and methods of identifying the functions of RapR6 gene and cellular  
35 pathways of RapR6 gene.

The invention, is based, in part, on the identification of the involvement of RapR6  
gene in rapamycin resistance and tumorigenesis using the random homozygous knockout

(RHKO) method. RHKO clones expressing a truncated fragment of a RapR6 protein  
5 reversibly exhibit resistance to rapamycin when the truncated fragment is expressed and  
sensitivity to rapamycin when the fragment is not expressed.

In the disclosure, resistance to rapamycin is often referred to. It will be apparent to  
a skilled person in the art that the disclosure is equally applicable to other rapamycin  
10 derivatives or analogs, such as, but not limited to, the rapamycin ester CCI-779.

For simplicity of discussion, the invention is described in the subsections below by  
way of examples for the human and murine RapR6 genes. However, the principles may be  
analogously applied to RapR6 genes of other species.

#### 15 5.1. DNA ENCODING RAPR6

The present invention provides a novel mammalian gene, the RapR6 gene. RapR6  
gene is identified as a gene involved in rapamycin resistance and tumorigenesis. The present  
invention also provides recombinant mammalian DNA molecules, cloned genes, or  
degenerate variants thereof, of a RapR6 gene which may involve in rapamycin resistance  
20 and tumorigenesis in a cell or organism.

##### 5.1.1. THE RAPR6 GENE

As used herein, "RapR6 gene" includes the genomic portion of DNA which is  
25 transcribed by RNA polymerase and encodes one or more RapR6 proteins. The RapR6  
gene may include a 5' untranslated region ("UTR"), introns, exons and a 3' UTR and  
regulatory sequences. The cDNA sequences of murine and human RapR6 genes and the  
encoded proteins are shown in FIGS. 2A-2B (SEQ ID NO:2 and SEQ ID NO:3) and FIGS.  
3A-3B (SEQ ID NO:10 and SEQ ID NO:11), respectively.

30 In preferred embodiments, the invention provides (a) a gene comprising the DNA  
sequence shown in FIGS. 2A and 3A, or comprised in the RHKO clone RapR6 in which a  
DNA construct is inserted in the intron between exon 1 and 2; (b) any DNA sequence that  
encodes the amino acid sequence shown in FIGS. 2B and 3B, or encoded by the gene in the  
RHKO clone RapR6 in which a DNA construct is inserted in the intron between exon 1 and  
35 2; (c) any DNA sequence that hybridizes to the complement of the DNA sequences that  
encode the amino acid sequence shown in FIGS. 2B and 3B, or encoded by the gene in the  
RHKO clone RapR6 in which a DNA construct is inserted in the intron between exon 1 and



2, under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M  
5 NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1 x  
SSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular  
Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York,  
at p. 2.10.3) and encodes a gene product functionally equivalent to a RapR6 gene product  
10 encoded by sequences shown in FIGS. 2A and 3A; and/or (d) any DNA sequence that  
hybridizes to the complement of the DNA sequences that encode the amino acid sequence  
shown in FIGS. 2B and 3B, or encoded by the gene in the RHKO clone RapR6 in which a  
DNA construct is inserted in the intron between exon 1 and 2, under less stringent  
conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2 x SSC/0.1% SDS  
15 at 42°C (Ausubel *et al.*, 1989, *supra*), yet which still encodes a functionally equivalent  
RapR6 gene product. As used herein, RapR6 genes also include degenerate variants of  
DNA sequences SEQ ID NO: 2 or 10.

The invention also provides exon and intron sequences comprised in a RapR6 gene.  
In one embodiment, the invention provides exons 1-6 of the murine RapR6 gene (SEQ ID  
20 NOS:4-9). In another embodiment, the invention provides the genomic sequence of a  
RapR6 gene comprised in a 4 kb plasmid carrying a 1.5 kb genomic DNA flanking the  
RHKO insertion site obtained by digestion of the RapR6 genomic DNA using HindIII.  
Sequences of three such plasmids (designated as RapR61, RapR62, and RapR63,  
respectively) are depicted in FIG. 1B.

25 The invention also includes portions of a RapR6 gene, *e.g.*, a portion encoding a  
fragment of a RapR6 protein. In a preferred embodiment, the invention provides a  
sequence flanking the insertion site of the knockout construct as illustrated in FIG. 1A  
(SEQ ID NO:1). In one embodiment, the invention provides a fragment of a RapR6 gene  
comprising the nucleotide region encoding a signal domain of a RapR6 gene product. In a  
30 specific embodiment, the invention provides a fragment of a human RapR6 gene  
comprising the nucleotide region encoding amino acids 1-17 of a human RapR6 protein, or  
its murine homolog. In one embodiment, the invention provides a fragment of a RapR6  
gene comprising the nucleotide region encoding a WD40 domain of a RapR6 gene product.  
In a specific embodiment, the invention provides a fragment of a human RapR6 gene  
35 comprising the nucleotide region encoding amino acids 24-63, 66-105, 108-154, 203-244,  
or 247-287 of a human RapR6 protein, or its murine homolog. In another embodiment, the  
invention provides a fragment of a RapR6 gene comprising the nucleotide region encoding

a transmembrane domain of a RapR6 gene product. In a specific embodiment, the invention provides a fragment of a murine RapR6 gene comprising the nucleotide region encoding amino acids 1-21 or 210-232 of a human RapR6 protein, or its murine homolog. In another embodiment, the invention provides a fragment of a RapR6 gene comprising the nucleotide region encoding a fragment not comprising a WD40 domain or a transmembrane domain of a RapR6 gene product. In a specific embodiment, the invention provides a fragment of a human RapR6 gene comprising the nucleotide region encoding amino acids 155-202, or any fragment thereof, of a human RapR6 protein, or its murine homolog. The invention also provides any sequence that is at least 30%, 50%, 70%, 90%, or 95% homologous to such fragments of a RapR6 gene.

The invention also provides nucleotide sequences comprising mutations in a RapR6 gene which cause a change in the amino acid sequence of the encoded protein. Exemplary SNP mutations are illustrated in FIG. 10.

The invention also provides nucleotide sequences which are comprised in a RapR6 gene and are at least 20, 25, 40, 60, 80, 100, 500, 1000 bases in length. Such sequences may be useful as probe sequences for monitoring expression of a RapR6 gene. The invention also provides nucleotide sequences which are comprised in a RapR6 gene and are at least 20, 50, 100, 500, 1000, 2000, 5000 bases in length. Such sequences may be useful for production of RapR6 peptides.

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the DNA sequences described in the preceding paragraphs. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, *e.g.*, to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may encode or act as RapR6 gene antisense molecules, useful, for example, in RapR6 gene regulation (for and/or as antisense primers in amplification reactions of RapR6 gene nucleic acid sequences. With respect to RapR6 gene regulation, such techniques can be used to regulate, for example, resistance to rapamycin. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for RapR6 gene regulation. Still further, such molecules may be used as components of diagnostic methods

whereby, for example, the presence of a particular RapR6 allele responsible for causing a  
5 RapR6 related disorder, such as rapamycin resistance and/or tumorigenesis, may be  
detected.

The invention also encompasses (a) DNA vectors that contain any of the foregoing  
RapR6 coding sequences and/or their complements (*i.e.*, antisense); (b) DNA expression  
10 vectors that contain any of the foregoing RapR6 coding sequences operatively associated  
with a regulatory element that directs the expression of the coding sequences; and (c)  
genetically engineered host cells that contain any of the foregoing RapR6 coding sequences  
operatively associated with a regulatory element that directs the expression of the coding  
sequences in the host cell. As used herein, regulatory elements include but are not limited  
15 to inducible and non-inducible promoters, enhancers, operators and other elements known  
to those skilled in the art that drive and regulate expression. Such regulatory elements  
include but are not limited to the cytomegalovirus hCMV immediate early gene, the early or  
late promoters of SV40 adenovirus, the *lac* system, the *trp* system, the *TAC* system, the  
*TRC* system, the major operator and promoter regions of phage A, the control regions of fd  
20 coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid  
phosphatase, and the promoters of the yeast  $\alpha$ -mating factors. The invention includes  
fragments of any of the DNA sequences disclosed herein. In preferred embodiments,  
RapR6 coding sequences are obtained by isolating the sequences flanking the insertion site  
in a RHKO clone.

25 In addition to the RapR6 gene sequences described above, homologs of such  
sequences present in other species can be identified and readily isolated, by molecular  
biological techniques well known in the art. Further, there can exist genes at other genetic  
loci within the genome that encode proteins which have extensive homology to one or more  
domains of the RapR6 gene product. These genes can also be identified via similar  
30 techniques.

With respect to the cloning of a RapR6 gene homolog in a species (*e.g.*, human), the  
isolated RapR6 gene sequences disclosed herein may be labeled and used to screen a cDNA  
library constructed from mRNA obtained from appropriate cells or tissues (*e.g.*, human  
35 MDCK cells) derived from the organism (*e.g.*, human) of interest. The hybridization  
conditions used should be of a lower stringency when the cDNA library is derived from an  
organism different from the type of organism from which the labeled sequence was derived.

Alternatively, the labeled fragment may be used to screen a genomic library derived from  
5 the organism of interest, again, using appropriately stringent conditions. Low stringency  
conditions are well known to those of skill in the art, and will vary predictably depending  
on the specific organisms from which the library and the labeled sequences are derived. For  
guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, Molecular  
Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel *et al.*, 1989,  
10 Current Protocols in Molecular Biology, Green Publishing Associates and Wiley  
Interscience, N.Y.

Further, a RapR6 gene homolog may be isolated from, for example, human nucleic  
acid, by performing PCR using two degenerate oligonucleotide primer pools designed on  
15 the basis of amino acid sequences within the RapR6 gene product disclosed herein. The  
template for the reaction may be cDNA obtained by reverse transcription of mRNA  
prepared from, for example, human or non-human cell lines or tissue known or suspected to  
express a RapR6 gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified  
20 sequences represent the sequences of a RapR6 gene nucleic acid sequence. The PCR  
fragment may then be used to isolate a full length cDNA clone by a variety of methods. For  
example, the amplified fragment may be labeled and used to screen a bacteriophage cDNA  
library. Alternatively, the labeled fragment may be used to isolate genomic clones via the  
screening of a genomic library.

25 PCR technology may also be utilized to isolate full length cDNA sequences. For  
example, RNA may be isolated, following standard procedures, from an appropriate cellular  
or tissue source (*i.e.*, one known, or suspected, to express the RapR6 gene, such as, for  
example, epithelia). A reverse transcription reaction may be performed on the RNA using  
30 an oligonucleotide primer specific for the most 5' end of the amplified fragment for the  
priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed"  
with guanines using a standard terminal transferase reaction, the hybrid may be digested  
with RNAase H, and second strand synthesis may then be primed with a poly-C primer.  
Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a  
35 review of cloning strategies which may be used, see *e.g.*, Sambrook *et al.*, 1989, *supra*.

RapR6 gene sequences may additionally be used to isolate mutant RapR6 gene  
alleles. Such mutant alleles may be isolated from individuals either known or proposed to

have a genotype which contributes to rapamycin resistance and/or tumorigenesis. Mutant  
5 alleles and mutant allele products may then be utilized in the therapeutic and diagnostic  
systems described below. Additionally, such RapR6 gene sequences can be used to detect  
RapR6 gene regulatory (*e.g.*, promoter) defects which can affect RapR6 expression and/or  
activity.

10 A cDNA of a mutant RapR6 gene may be isolated, for example, by using PCR, a  
technique which is well known to those of skill in the art. In this case, the first cDNA  
strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated  
from tissue known or suspected to be expressed in an individual putatively carrying the  
mutant RapR6 allele, and by extending the new strand with reverse transcriptase. The  
15 second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes  
specifically to the 5' end of the normal gene. Using these two primers, the product is then  
amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis  
through methods well known to those of skill in the art. By comparing the DNA sequence  
of the mutant RapR6 allele to that of the normal RapR6 allele, the mutation(s) responsible  
20 for the loss or alteration of function of the mutant RapR6 gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an  
individual suspected of or known to carry the mutant RapR6 allele, or a cDNA library can  
be constructed using RNA from a tissue known, or suspected, to express the mutant RapR6  
allele. The normal RapR6 gene or any suitable fragment thereof may then be labeled and  
25 used as a probe to identify the corresponding mutant RapR6 allele in such libraries. Clones  
containing the mutant RapR6 gene sequences may then be purified and subjected to  
sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized  
30 from, for example, RNA isolated from a tissue known, or suspected, to express a mutant  
RapR6 allele in an individual suspected of or known to carry such a mutant allele. In this  
manner, gene products made by the putatively mutant tissue may be expressed and screened  
using standard antibody screening techniques in conjunction with antibodies raised against  
the normal RapR6 gene product, as described, below, in Section 5.3. (For screening  
35 techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory  
Manual", Cold Spring Harbor Press, Cold Spring Harbor.) In cases where a RapR6  
mutation results in an expressed gene product with altered function (*e.g.*, as a result of a

missense or a frameshift mutation), a polyclonal set of anti-RapR6 gene product antibodies  
5 are likely to cross-react with the mutant RapR6 gene product. Library clones detected via  
their reaction with such labeled antibodies can be purified and subjected to sequence  
analysis according to methods well known to those of skill in the art.

#### 5.1.2. METHODS OF IDENTIFYING A GENE INVOLVED IN RAPAMYCIN RESISTANCE AND TUMORGENESIS

10 The involvement of a gene in rapamycin resistance and/or tumorigenesis can be  
identified by introducing randomly into the genome or a suitable cell, e.g., an N2a cell, a  
DNA construct (i.e., the knockout construct) such that a gene is activated or inactivated, and  
screening for resultant clones which exhibit phenotypic changes in rapamycin resistance  
15 and/or tumorigenesis. Any mammalian cells include but are not limited to N2a, NT2, NT22,  
CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38 can be used. Preferably, the cell  
line used is a rapamycin sensitive cell line, and the resultant clones are rapamycin resistant.  
Alternatively, a rapamycin resistant cell line can be used as the starting cell line, and the  
resultant clones are rapamycin sensitive.

20 In a preferred embodiment, a rapamycin resistance and/or tumorigenesis clone  
contains a gene which is knocked out by the random homozygous knockout (RHKO)  
process (see, e.g., U.S. Patent Nos. 5,679,523; 5,807,995; 5,891,668; and 6,248,523; Li et  
al., 1996, Cell 85:319-329; PCT publication no. WO 03/027260, each of which is  
25 incorporated herein by reference in its entirety). In a RHKO clone, multiple alleles of a  
gene at a random chromosomal locus in the genome of a mammalian cell are inactivated  
concurrently. In another preferred embodiment, a rapamycin resistance and/or  
tumorigenesis clone contains an insertion of a suitable construct at a genomic locus such that  
the expression of a gene at the locus is activated or enhanced. In still another preferred  
embodiment, a rapamycin resistance and/or tumorigenesis clone contains an insertion of a  
30 suitable construct at a genomic locus such that a portion of a gene at the locus is  
overexpressed.

Preferably, a knockout construct (or gene search construct) comprising a selection  
marker sequence and a regulated promoter responsive to a transactivation factor and  
35 controlling the expression of the selection marker sequence is inserted into the genome of a  
selected cell line using a standard method known in the art, e.g., transefection or retroviral  
infection. In a preferred embodiment, a retroviral gene search vector comprising the

knockout construct, viral genes and regulatory elements, and a Cre/Lox site specific  
5 recombination system is used to introduce the knockout construct into the genome of a cell  
(FIG. 9C). The Cre/Lox system allows the use of the retroviral elements for infection and  
the subsequent removal of the retroviral elements from chromosomal DNA after vector  
integration. Such a retroviral gene search vector allows highly efficient retroviral  
10 integration in target cells (up to 90%), and at the same time eliminate retroviral interference  
of selection marker gene activity and RNA transcription. The selection marker gene in the  
gene search vector can be a fusion gene consisting of a neomycin resistance gene and a  
bacterial  $\beta$ -galactosidase gene. In a preferred embodiment, the selection marker gene  
consists of a neomycin resistance gene or a puromycin resistance gene. In another preferred  
15 embodiment, the selection marker gene consists of a neomycin resistance gene or a  
puromycin resistance gene and a gene encoding a fluorescence protein such as a green  
fluorescence protein, e.g., a GFP-Neo or a GFP-Puro. Incorporation of fluorescence  
proteins allows direct detection of selection marker gene activity in living cells and direct  
isolation of positive cells by FACS (Fluorescence Activated Cell Sorter) without having to  
20 stain the cells. Preferably, the gene search vector further comprises a gene splicing element  
and/or an independent translation initiation signal. In one embodiment, a viral internal  
ribosomal entry site (IRES) is inserted into the selection marker gene to allow efficient  
translation in all three reading frames. More preferably, another DNA construct which  
includes a promoter, e.g., an SMV promoter, and a nucleotide sequence encoding the  
transactivator and under the control of the promoter is also inserted into the genome of the  
25 cells to allow production of the transactivator.

Preferably, the regulated promoter provides a rheostat genetic on/off switch. For  
example, either a Tet-on<sup>TM</sup> or a Tet-off<sup>TM</sup> system (Clontech, Palo Alto, CA, see, e.g.,  
<http://www.clontech.com/products/literature/pdf/productlists/tetprodlist.pdf> and  
30 <http://www.clontech.com/products/literature/pdf/brochures/TetBR.pdf>, accessed on May 3,  
2002) can be used for this purpose. In a preferred embodiment, a Tet-off<sup>TM</sup> system  
(Clontech) is used. In a Tet-off<sup>TM</sup> system the regulated promoter is a tetracycline regulated  
promoter which can be activated by a tetracycline regulated transactivator (see, e.g., Gossen  
et al, 1995, Science 268:1766-1769; Gossen et al., 1992, Proc. Natl. Acad. Sci. USA  
89:5547-5551). The transactivator binds to tetracycline regulated promoter in the absence  
35 of tetracycline but not in the presence of tetracycline. Therefore, gene expression is kept off  
in the presence of tetracycline, whereas gene transcription is activated in the absence of  
tetracycline. Incorporation of a tetracycline regulation system into RHKO allows

transcription of RNA, e.g., transcription of antisense RNA, to be turned on or off by  
5 removing or adding tetracycline in the cell culture medium, and also allows the rheostat  
regulation of RNA production by controlling the amount of tetracycline in the culture  
medium. This enables rapid validation of the antisense RNA effect by adding and removing  
tetracycline or a derivative or analog thereof, and determination of gene function under  
rheostat regulation.

10

The knockout construct can preferably contain a rapid cloning element comprising a  
bacterial plasmid replication origin, e.g., an Ori, and a bacterial selection marker, e.g., a  
chloramphenicol resistance gene for rapid and direct isolation of target genes. The  
chromosomal gene, the selection marker sequence, the plasmid replication origin, and the  
15 bacterial selection marker are transcribed as a single fusion messenger RNA, which is  
converted to double strand cDNA and circularized to a circular plasmid. The resulting  
circular plasmids are transformed into bacteria and rapidly amplified without additional  
gene cloning. The chromosomal genes are identified by sequencing the amplified plasmids.  
Alternatively the genomic DNA can be digested with restriction enzyme (such as Hind III,  
20 BamH1), recirculized by self-ligation and are transformed into bacteria and rapidly  
amplified. The genomic DNA flanking the knockout vector can be rapidly cloned directly  
by this strategy. Any bacterial plasmid replication origin, such as but not limited to Ori,  
colEI, pSC101, pUC, or f1 phage ori, can be used. Any bacterial selection markers, such as  
but not limited to, chloramphenicol, ampicillin, tetracycline, or kanamycin, can be used in  
25 the present invention.

In one embodiment, the construct is inserted into the open reading frame region of a  
genomic locus such that the transcription initiation sequence in the knockout construct is  
oriented for antisense RNA transcription in the direction away from the selection marker  
region sequence such that when activated by the transactivation factor, it initiates antisense  
30 RNA transcription extending from the knockout construct into the chromosomal locus  
flanking the knockout construct at its 5' end. Thus, although only one allele of the gene is  
knocked out, antisense RNA transcripts inactivate the other allele or alleles. Cells contain  
the knockout construct, i.e., the RHKO clones, is selected based on the presence of activity  
of the selection marker. RHKO clones in which a gene involved in rapamycin resistance is  
35 inactivated are then subjected to rapamycin treatment, and rapamycin resistance RHKO  
clones are identified. In a preferred embodiment, the insertion site is in an exon of the gene.  
In another preferred embodiment, the insertion site is in an intron.



In another embodiment, the construct is inserted in front of an endogenous promoter  
 5 such that the transcription initiation sequence in the knockout construct is oriented for  
 activation or enhancement of the expression of the gene controlled by the endogenous  
 promoter.

In still another embodiment, the knockout construct is inserted into the open reading  
 10 frame region of a genomic locus such that the transcription initiation sequence in the  
 knockout construct is oriented for transcription of a portion of the open reading frame of the  
 gene, thereby activating expression or overexpressing the entire or a portion of sequence  
 which encodes the protein. In one embodiment, the construct is inserted before the ATG  
 codon, thereby activating production or overproduction of the entire encoded protein. In  
 15 another embodiment, the construct is inserted after the ATG codon, thereby activating  
 production or overproduction of a fragment of the encoded protein.

In one embodiment, a rapamycin resistance clone, e.g., a RHKO clone, is identified  
 by treating the obtained clones with a suitable concentration of rapamycin for a suitable  
 period of time. In a preferred embodiment, when an N2a cell line is used to generate the  
 20 clone, a rapamycin resistance clone is identified by treating the obtained clones with 1  $\mu$ M  
 of rapamycin for 14 days. RHKO induced rapamycin resistance is further verified by  
 selecting rapamycin resistant clones which exhibit reversible rapamycin resistance. As used  
 herein, reversibility R is defined as a at least two fold reduction of rapamycin's inhibitory  
 effect when the expression of the knockout construct is suppressed, e.g., when the  
 25 expression of the transactivation factor is suppressed. For example, reversibility R may be  
 defined as

$$R = \frac{\% \text{ Inhibition by Rapamycin when the gene search construct is on}}{\% \text{ Inhibition by Rapamycin when the gene search construct is off}} - 2 \quad (1)$$

In a preferred embodiment, the reversibility of rapamycin resistance is assayed by  
 30 comparing rapamycin resistance in the presence and absence of the transactivation factor.  
 RHKO clones that is rapamycin resistant in the presence of the transactivation factor and  
 rapamycin sensitive in the absence of the transactivation factor are identified. In another  
 preferred embodiment of the invention, a second construct comprising a marker gene and  
 the transactivation factor that activate the transcription initiation sequence of the knockout  
 35 construct operably linked to a regulated promoter is also introduced into the genome of the  
 selected cell line. The activation of the knockout construct can then be regulated by  
 activating or suppressing the regulated promoter in the second construct. In another

preferred embodiment, the RHKO clones are further assayed using any standard method  
5 known in the art, e.g., Southern blotting, such that clones that contain a single copy of the  
knockout construct can be identified.

Once RHKO clones which exhibit reversible rapamycin resistance are identified, the  
genomic DNA sequence flanking the integration site of the knockout construct can be  
10 obtained and sequenced by any standard method known in the art. Preferably, the flanking  
genomic sequence obtained and sequenced is at least about 500 bases in length. More  
preferably, the flanking genomic sequence obtained and sequenced is at least about 1000  
bases in length. Still more preferably, the flanking genomic sequence obtained and  
sequenced is between 500 to 5000 bases in length. Still more preferably, the flanking  
15 genomic sequence obtained and sequenced is in the range of 1000 to 3000 bases in length.  
In one embodiment, the entire open reading frame is obtained and sequenced. In another  
embodiment, the regulatory sequence is obtained and sequenced. In a preferred  
embodiment, more than one sequences for a clone may be obtained, and a consensus  
sequence is determined using any standard method known in the art. Most preferably, the  
20 regulatory sequences and the entire open reading frame are obtained and sequenced.

The obtained sequence can then be used as the query sequence to search one or more  
databases. Any method known in the art can be used for this purpose. The methods can  
make use of any sequence information available for the organism, including but not limited  
to, the genomic sequence data, the protein sequence data, mRNA sequence data, and EST  
25 data in conjunction with computational sequence analysis tools to identify the coding  
regions and or regulatory sequences in the genome of the organism. One skilled person in  
the art will be able to choose one or more methods, e.g., BLAST, and one or more  
appropriate databases, e.g., Ensembl, GenBank, etc. In a preferred embodiment, the  
structure of the gene, e.g., exon and/or intron sequences, is determined by comparing the  
30 expressed mRNA sequences or cDNAs or ESTs derived therefrom to the genomic sequence  
of the organism.

In one embodiment, the invention provides an RHKO clone, RapR6, which is a  
murine N2a cell containing a knockout construct inserted in the intron between exon 1 and  
35 exon 2 of a RapR6 gene and oriented for sense RNA transcription. Thus, when the  
expression of the knockout construct is activated, e.g., in the presence of a transactivator, a  
truncated fragment of the RapR6 protein is expressed in the RapR6 clone. FIGS. 8A-8E

show that the RapR6 clone exhibits reversible rapamycin resistance and tumorigenesis. FIG. 1A shows a fragment of the genomic sequence flanking of the insertion site.

## 5.2. RAPR6 GENE PRODUCTS AND CELL LINES THAT EXPRESS RAPR6

The present invention provides RapR6 gene products, e.g., proteins or fragments thereof, cell lines that are engineered to express RapR6, as well as transgenic animals that are engineered to express RapR6.

### 5.2.1. RAPR6 GENE PRODUCTS

RapR6 gene products, or peptide fragments thereof, can be prepared for a variety of uses. For example, such gene products, or peptide fragments thereof, can be used for the generation of antibodies, in diagnostic assays, or for the identification of other cellular gene products involved in the regulation of expression and/or activities of RapR6 gene.

The amino acid sequences depicted in FIGS. 2B and 3B represent RapR6 gene products. The RapR6 gene product, sometimes referred to herein as an "RapR6 protein or polypeptide", may additionally include those gene products encoded by the RapR6 gene sequences described in Section 5.1, above. In one embodiment, the invention provides a 289 amino acid murine RapR6 protein (PI: 8.53; MW: 32.25 kDa). In another embodiment, the invention provides a 287 amino acid human RapR6 protein (PI: 8.64; MW: 32.17 kDa).

In addition, RapR6 gene products may include proteins that represent functionally equivalent gene products. Such an equivalent RapR6 gene product may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by the RapR6 gene sequences described, above, in Section 5.1, but which result in a silent change, thus producing a functionally equivalent RapR6 gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

“Functionally equivalent”, as utilized herein, refers to a protein capable of exhibiting  
5 a substantially similar *in vivo* activity as the endogenous RapR6 gene products encoded by  
the RapR6 gene sequences described in Section 5.1, above. The *in vivo* activity of the  
RapR6 gene product, as used herein, refers to the ability of the RapR6 gene product, when  
present in an appropriate cell type, to ameliorate, prevent or delay the appearance of the  
RapR6 abnormal phenotype.

10 The invention also includes fragments of a RapR6 protein. In one embodiment, the  
invention provides a fragment of a RapR6 gene product comprising a signal domain. In a  
specific embodiment, the invention provides a fragment of a human RapR6 gene product  
comprising amino acids 1-17 of a human RapR6 protein, or its murine homolog. In one  
15 embodiment, the invention provides a fragment of a RapR6 gene product comprising a  
WD40 domain. In a specific embodiment, the invention provides a fragment of a human  
RapR6 gene product comprising amino acids 24-63, 66-105, 108-154, 203-244, or 247-287  
of a human RapR6 protein, or its murine homolog. In another embodiment, the invention  
provides a fragment of a RapR6 gene product comprising a transmembrane domain. In a  
20 specific embodiment, the invention provides a fragment of a human RapR6 gene product  
comprising amino acids 1-21 or 210-232 of a human RapR6 protein, or its murine homolog.  
In another embodiment, the invention provides a fragment of a RapR6 gene product not  
comprising a WD40 domain or a transmembrane domain. In a specific embodiment, the  
invention provides a fragment of a human RapR6 gene product comprising amino acids  
25 155-202, or any fragment thereof, of a human RapR6 protein, or its murine homolog. The  
invention also provides any sequence that is at least 30%, 50%, 70%, 90%, or 95%  
homologous such fragments of a RapR6 protein. The invention also provides peptides  
which are comprised in a RapR6 gene product and are at least 5, 10, 20, 50, 100 amino  
acids in length.

30 The RapR6 gene products or peptide fragments thereof, may be produced by  
recombinant DNA technology using techniques well known in the art. Thus, methods for  
preparing the RapR6 gene polypeptides and peptides of the invention by expressing nucleic  
acid containing RapR6 gene sequences are described herein. Methods which are well  
known to those skilled in the art can be used to construct expression vectors containing  
35 RapR6 gene product coding sequences and appropriate transcriptional and translational  
control signals. These methods include, for example, *in vitro* recombinant DNA  
techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the

techniques described in Sambrook *et al.*, 1989, *supra*, and Ausubel *et al.*, 1989, *supra*.

- 5 Alternatively, RNA capable of encoding RapR6 gene product sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated herein by reference in its entirety.

#### 10 5.2.2. CELL LINES THAT EXPRESS RAPR6

- A variety of host-expression vector systems may be utilized to express the RapR6 gene coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate
- 15 nucleotide coding sequences, exhibit the RapR6 gene product of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing RapR6 gene product coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing
- 20 the RapR6 gene product coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the RapR6 gene product coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing RapR6 gene product
- 25 coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3, N2a) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

- 30 In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the RapR6 gene product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of RapR6 protein or for raising antibodies to RapR6 protein, for example, vectors which direct the expression of high levels of fusion protein products
- 35 that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO J. 2:1791), in which the RapR6 gene product coding sequence may be ligated individually into the vector in frame

with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye &  
5 Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol.  
Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign  
polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such  
fusion proteins are soluble and can easily be purified from lysed cells by adsorption to  
glutathione-agarose beads followed by elution in the presence of free glutathione. The  
10 pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that  
the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is  
used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells.  
15 The RapR6 gene coding sequence may be cloned individually into non-essential regions  
(for example the polyhedrin gene) of the virus and placed under control of an AcNPV  
promoter (for example the polyhedrin promoter). Successful insertion of RapR6 gene  
coding sequence will result in inactivation of the polyhedrin gene and production of non-  
occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the  
20 polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda*  
cells in which the inserted gene is expressed. (*E.g.*, see Smith *et al.*, 1983, J. Virol. 46: 584;  
Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be  
utilized. In cases where an adenovirus is used as an expression vector, the RapR6 gene  
25 coding sequence of interest may be ligated to an adenovirus transcription/translation control  
complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may  
then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in  
a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a  
recombinant virus that is viable and capable of expressing RapR6 gene product in infected  
30 hosts. (*E.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659).  
Specific initiation signals may also be required for efficient translation of inserted RapR6  
gene product coding sequences. These signals include the ATG initiation codon and  
adjacent sequences. In cases where an entire RapR6 gene, including its own initiation  
codon and adjacent sequences, is inserted into the appropriate expression vector, no  
35 additional translational control signals may be needed. However, in cases where only a  
portion of the RapR6 gene coding sequence is inserted, exogenous translational control  
signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the

initiation codon must be in phase with the reading frame of the desired coding sequence to  
5 ensure translation of the entire insert. These exogenous translational control signals and  
initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of  
expression may be enhanced by the inclusion of appropriate transcription enhancer  
elements, transcription terminators, etc. (see Bittner *et al.*, 1987, *Methods in Enzymol.*  
153:516-544).

10 In addition, a host cell strain may be chosen which modulates the expression of the  
inserted sequences, or modifies and processes the gene product in the specific fashion  
desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein  
products may be important for the function of the protein. Different host cells have  
15 characteristic and specific mechanisms for the post-translational processing and  
modification of proteins and gene products. Appropriate cell lines or host systems can be  
chosen to ensure the correct modification and processing of the foreign protein expressed.  
To this end, eukaryotic host cells which possess the cellular machinery for proper  
processing of the primary transcript, glycosylation, and phosphorylation of the gene product  
20 may be used. Such mammalian host cells include but are not limited to CHO, VERO,  
BHK, HeLa, COS, MDCK, 293, 3T3, WI38.

For long-term, high-yield production of recombinant proteins, stable expression is  
preferred. For example, cell lines which stably express the RapR6 gene product may be  
engineered. Rather than using expression vectors which contain viral origins of replication,  
25 host cells can be transformed with DNA controlled by appropriate expression control  
elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation  
sites, etc.), and a selectable marker. Following the introduction of the foreign DNA,  
engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are  
switched to a selective media. The selectable marker in the recombinant plasmid confers  
30 resistance to the selection and allows cells to stably integrate the plasmid into their  
chromosomes and grow to form foci which in turn can be cloned and expanded into cell  
lines. This method may advantageously be used to engineer cell lines which express the  
RapR6 gene product. Such engineered cell lines may be particularly useful in screening and  
evaluation of compounds that affect the endogenous activity of the RapR6 gene product.

35 In another embodiment, the expression characteristics of an endogenous gene (*e.g.*, a  
RapR6 gene) within a cell, cell line or microorganism may be modified by inserting a DNA

regulatory element heterologous to the endogenous gene of interest into the genome of a  
5 cell, stable cell line or cloned microorganism such that the inserted regulatory element is  
operatively linked with the endogenous gene (*e.g.*, a RapR6 gene) and controls, modulates,  
activates, or inhibits the endogenous gene. For example, endogenous RapR6 genes which  
are normally "transcriptionally silent", *i.e.*, a RapR6 gene which is normally not expressed,  
or is expressed only at very low levels in a cell line or microorganism, may be activated by  
10 inserting a regulatory element which is capable of promoting the expression of the gene  
product in that cell line or microorganism. Alternatively, transcriptionally silent,  
endogenous RapR6 genes may be activated by insertion of a promiscuous regulatory  
element that works across cell types.

15 A heterologous regulatory element may be inserted into a stable cell line or cloned  
microorganism, such that it is operatively linked with and activates or inhibits expression of  
endogenous RapR6 genes, using techniques, such as targeted homologous recombination,  
which are well known to those of skill in the art, and described *e.g.*, in Chappel, U.S. Patent  
No. 5,272,071; PCT Publication No. WO 91/06667 published May 16, 1991; Skoultchi,  
20 U.S. Patent No. 5,981,214; and Treco *et al* U.S. Patent No. 5,968,502 and PCT Publication  
No. WO 94/12650 published June 9, 1994. Alternatively, non-targeted, *e.g.* non-  
homologous recombination techniques may be used which are well-known to those of skill  
in the art and described, *e.g.*, in PCT Publication No. WO 99/15650 published April 1,  
1999.

25 RapR6 gene activation (or inactivation) may also be accomplished using designer  
transcription factors using techniques well known in the art. Briefly, a designer zinc finger  
protein transcription factor (ZFP-TF) is made which is specific for a regulatory region of  
the RapR6 gene to be activated or inactivated. A construct encoding this designer ZFP-TF  
is then provided to a host cell in which the RapR6 gene is to be controlled. The construct  
30 directs the expression of the designer ZFP-TF protein, which in turn specifically modulates  
the expression of the endogenous RapR6 gene. The following references relate to various  
aspects of this approach in further detail: Wang & Pabo, 1999, Proc. Natl. Acad. Sci. USA  
96, 9568; Berg, 1997, Nature Biotechnol. 15, 323; Greisman & Pabo, 1997, Science 275,  
657; Berg & Shi, 1996, Science 271, 1081; Rebar & Pabo, 1994, Science 263, 671;  
35 Rhodes & Klug, 1993, Scientific American 269, 56; Pavletich & Pabo, 1991, Science 252,  
809; Liu *et al.*, 2001, J. Biol. Chem. 276, 11323; Zhang *et al.*, 2000, J. Biol. Chem. 275,  
33850; Beerli *et al.*, 2000, Proc. Natl. Acad. Sci. USA 97, 1495; Kang *et al.*, 2000, J. Biol.



Chem. 275, 8742; Beerli et al., 1998, Proc. Natl. Acad. Sci. USA 95, 14628; Kim & Pabo, 5 1998, Proc. Natl. Acad. Sci. USA 95, 2812; Choo et al., 1997, J. Mol. Biol. 273, 525; Kim & Pabo, 1997, J. Biol. Chem. 272, 29795; Liu et al, 1997, Proc. Natl. Acad. Sci. USA 94, 5525; Kim et al, 1997, Proc. Natl. Acad. Sci. USA 94, 3616; Kikyo et al., 2000, Science 289, 2360; Robertson & Wolffe, 2000, Nature Reviews 1, 11; and Gregory, 2001, Curr. Opin. Genet. Devt. 11, 142.

10 A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, Cell 22:817) genes  
15 can be employed in *tk<sup>-</sup>*, *hgp<sup>r</sup>* or *ap<sup>r</sup>* cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: *dhfr*, which confers resistance to methotrexate (Wigler, *et al.*, 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers  
20 resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, J. Mol. Biol. 150:1); and *hygro*, which confers resistance to hygromycin (Santerre, *et al.*, 1984, Gene 30:147).

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by  
25 Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia  
30 virus are loaded onto Ni<sup>2+</sup>-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

#### 5.2.3. CELL LINES THAT EXPRESS A TRUNCATED FRAGMENT OF A RAPR6 PROTEIN REVERSIBLY

35 The invention also provides cell lines in which a portion of the RapR6 gene can be reversibly expressed, as produced by the method described in Section 5.1.2. Such cell lines are useful, e.g., for identifying RapR6 related cellular pathways and/or for screening for

agents that modulate the expression of RapR6 gene and/or the interactions of RapR6 gene  
5 with other molecules.

#### 5.2.4. TRANSGENIC ANIMALS THAT EXPRESS RAPR6

The RapR6 gene products can also be expressed in transgenic animals. Animals of  
any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs,  
10 goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to  
generate RapR6 transgenic animals.

Any technique known in the art may be used to introduce the RapR6 gene transgene  
into animals to produce the founder lines of transgenic animals. Such techniques include,  
15 but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S.  
Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*,  
1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells  
(Thompson *et al.*, 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell.  
Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-  
20 723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl.  
Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the RapR6  
transgene in all their cells, as well as animals which carry the transgene in some, but not all  
their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in  
25 concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also  
be selectively introduced into and activated in a particular cell type by following, for  
example, the teaching of Lasko *et al.* (Lasko, M. *et al.*, 1992, Proc. Natl. Acad. Sci. USA  
89: 6232-6236). The regulatory sequences required for such a cell-type specific activation  
will depend upon the particular cell type of interest, and will be apparent to those of skill in  
30 the art. When it is desired that the RapR6 transgene be integrated into the chromosomal site  
of the endogenous RapR6 gene, gene targeting is preferred. Briefly, when such a technique  
is to be utilized, vectors containing some nucleotide sequences homologous to the  
endogenous RapR6 gene are designed for the purpose of integrating, via homologous  
recombination with chromosomal sequences, into and disrupting the function of the  
35 nucleotide sequence of the endogenous RapR6 gene. The transgene may also be selectively  
introduced into a particular cell type, thus inactivating the endogenous RapR6 gene in only  
that cell type, by following, for example, the teaching of Gu *et al.* (Gu, *et al.*, 1994, Science

265: 103-106). The regulatory sequences required for such a cell-type specific inactivation  
5 will depend upon the particular cell type of interest, and will be apparent to those of skill in  
the art.

Once transgenic animals have been generated, the expression of the recombinant  
RapR6 gene may be assayed utilizing standard techniques. Initial screening may be  
10 accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to  
assay whether integration of the transgene has taken place. The level of mRNA expression  
of the transgene in the tissues of the transgenic animals may also be assessed using  
techniques which include but are not limited to Northern blot analysis of tissue samples  
obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of RapR6  
15 gene-expressing tissue, may also be evaluated immunocytochemically using antibodies  
specific for the RapR6 transgene product.

### 5.3. ANTIBODIES TO RAPR6 GENE PRODUCTS

The present invention provides antibodies that bind to RapR6 gene products, e.g., a  
20 human RapR6 protein or a fragment thereof. Such antibodies may include, but are not  
limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric  
antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced  
by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding  
fragments of any of the above. Such antibodies may be used, for example, in the detection  
25 of a RapR6 gene product in an biological sample and may, therefore, be utilized as part of a  
diagnostic or prognostic technique whereby patients may be tested for abnormal levels of  
RapR6 gene products, and/or for the presence of abnormal forms of the such gene products.  
Such antibodies may also be utilized in conjunction with, for example, compound screening  
schemes, as described, below, in Section 5.4.2, for the evaluation of the effect of test  
30 compounds on RapR6 gene product levels and/or activity. Additionally, such antibodies  
can be used in conjunction with the gene therapy techniques described, below, in Section  
5.4.3, to, for example, evaluate the normal and/or engineered RapR6-expressing cells prior  
to their introduction into the patient.

35 Anti-RapR6 gene product antibodies may also be used for the inhibition of abnormal  
RapR6 gene product activity. Anti-RapR6 gene product antibodies may additionally be  
used for the inhibition of abnormal RapR6 gene product activity. Thus, such antibodies

may, therefore, be utilized as part of treatment methods of a disease resulting from defective  
5 regulation of RapR6 gene expression and/or abnormal RapR6 gene product activity.

5.3.1. METHODS OF SCREENING FOR ANTIBODIES DIRECTED TO RAPR6  
PROTEIN AND DOMAINS/FRAGMENTS OF RAPR6 PROTEIN

The present invention provides methods for screening for antibodies that bind to  
10 RapR6 protein. The methods involve screening for antibodies using an appropriate  
polypeptides of a RapR6 protein. Any fragment of the RapR6 protein, e.g., those described  
in Section 5.2., can be used to raise the antibody of the invention.

Screening for desired antibody can be accomplished by techniques known in the art.  
In one embodiment, antibodies which recognize a specific domain of a RapR6, generated  
15 hybridomas are assayed for a product which binds to a RapR6 fragment containing such  
domain.

In another embodiment, an antibody directed against RapR6 protein or a  
fragment/polypeptide of a RapR6 protein can be identified and isolated by screening a  
20 recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library)  
with the RapR6 protein or a fragment/polypeptide of a RapR6 protein. Kits for generating  
and screening phage display libraries are commercially available (e.g., Pharmacia  
Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene antigen  
SurfZAP™ Phage Display Kit, Catalog No. 240612). Additionally, examples of methods  
25 and reagents particularly amenable for use in generating and screening antibody display  
library can be found in, for example, U.S. Patent Nos. 5,223,409 and 5,514,548; PCT  
Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No.  
WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288;  
PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication  
30 No. WO 90/02809; Fuchs *et al.*, 1991, Bio/Technology 9:1370-1372; Hay *et al.*, 1992,  
Hum. Antibod. Hybridomas 3:81-85; Huse *et al.*, 1989, Science 246:1275-1281; Griffiths *et al.*,  
1993, EMBO J. 12:725-734. A phage display library permits selection of desired  
antibody or antibodies from a very large repertoire of specificities. An additional advantage  
of a phage display library is that the nucleic acids encoding the selected antibodies can be  
35 obtained conveniently, thereby facilitating subsequent construction of expression vectors.

For selection of an antibody that specifically binds a RapR6 but which does not  
specifically bind a related cellular protein by any of the above mentioned methods of this

section, one can select on the basis of positive binding to the RapR6 and a lack of binding  
5 to the related cellular protein. In a preferred embodiment, the sequence of a RapR6  
fragment used for the selection of antibodies is a sequence not comprised by other RapR6  
whose activities are to be preserved.

In one embodiment, the invention provides an antibody that binds the 289 amino  
10 acid murine RapR6 protein (SEQ ID NO:3). In another embodiment, the invention  
provides an antibody that binds the 287 amino acid human RapR6 protein (SEQ ID NO:11).  
In one embodiment, the invention provides an antibody that binds a polypeptide comprising  
a signal domain, a WD40 domain, or a transmembrane domain of a murine or human  
RapR6 protein. In specific embodiments, the invention provides an antibody that binds a  
15 polypeptide comprising amino acids 1-17, 24-63, 66-105, 108-154, 203-244, 247-287, 1-21,  
210-232 or 155-202, or any fragment thereof, of a human RapR6 protein; or a polypeptide  
comprising the corresponding murine homologs. The invention also provides an antibody  
that binds any sequence that is at least 30%, 50%, 70%, 90%, or 95% homologous to such  
fragments of a RapR6 protein.

20

### 5.3.2. METHODS OF PRODUCTION OF ANTIBODIES

Described herein are methods for the production of antibodies capable of  
specifically recognizing a sequence of a RapR6 gene or one or more RapR6 gene product  
25 epitopes or epitopes of conserved variants or peptide fragments of the RapR6 gene  
products.

For the production of antibodies against a RapR6 gene product, various host animals  
may be immunized by injection with a RapR6 gene product, or a portion thereof. Such host  
animals may include but are not limited to rabbits, mice, and rats, to name but a few.  
30 Various adjuvants may be used to increase the immunological response, depending on the  
host species, including but not limited to Freund's (complete and incomplete), mineral gels  
such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic  
polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol,  
and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and  
35 *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived  
from the sera of animals immunized with an antigen, such as a RapR6 gene product, or an

antigenic functional derivative thereof. For the production of polyclonal antibodies, host  
5 animals such as those described above, may be immunized by injection with RapR6 gene  
product supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a  
particular antigen, such as a RapR6 gene product, or an antigenic functional derivative  
10 thereof, may be obtained by any technique which provides for the production of antibody  
molecules by continuous cell lines in culture. These include, but are not limited to, the  
hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Patent  
No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, Immunology  
Today 4:72; Cole *et al.*, 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-  
15 hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies And Cancer Therapy, Alan  
R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including  
IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of  
this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in*  
*vivo* makes this the presently preferred method of production.

20 In addition, techniques developed for the production of "chimeric antibodies"  
(Morrison *et al.*, 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.*, 1984,  
Nature, 312:604-608; Takeda *et al.*, 1985, Nature, 314:452-454) by splicing the genes from  
a mouse antibody molecule of appropriate antigen specificity together with genes from a  
human antibody molecule of appropriate biological activity can be used. A chimeric  
25 antibody is a molecule in which different portions are derived from different animal species,  
such as those having a variable region derived from a murine mAb and a human  
immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies  
30 (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl.  
Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989, Nature 334:544-546) can be adapted  
to produce single chain antibodies against RapR6 gene products. Single chain antibodies  
are formed by linking the heavy and light chain fragments of the Fv region via an amino  
acid bridge, resulting in a single chain polypeptide.

35 Antibody fragments which recognize specific epitopes may be generated by known  
techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub>  
fragments which can be produced by pepsin digestion of the antibody molecule and the Fab

fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

#### 5.4. USES OF RAPR6 GENE, GENE PRODUCTS, CELL LINES AND ANTIBODIES

The invention provides methods and compositions for utilizing the RapR6 gene, product and antibodies for identifying proteins or other molecules that interact with RapR6 gene or protein. The invention also provides methods and compositions for utilizing the RapR6 gene, product and antibodies for screening for agents that RapR6 expression or modulating interaction of RapR6 gene or protein with other proteins or molecules. The invention further provides methods and compositions for utilizing the RapR6 gene, product and antibodies for screening for agents that are useful in regulating rapamycin resistance and/or tumorigenesis in a cell or organism. The invention also provides methods and compositions for utilizing RapR6 gene, product and antibodies for diagnosing RapR6 mediated rapamycin resistance and/or tumorigenesis, and for treatment of diseases in conjunction with a rapamycin therapy.

##### 5.4.1. METHODS OF DETERMINING PROTEINS OR OTHER MOLECULES THAT INTERACT WITH RAPR6 GENE OR GENE PRODUCT

Any method suitable for detecting protein-protein interactions may be employed for identifying RapR6 protein-cellular protein interactions. The interaction between RapR6 gene and other cellular molecules, e.g., interaction between RapR6 and its regulators may also be determined using methods known in the art.

Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Utilizing procedures such as these allows for the identification of cellular proteins which interact with RapR6 gene products. Once isolated, such a cellular protein can be identified and can, in turn, be used, in conjunction with standard techniques, to identify proteins it interacts with. For example, at least a portion of the amino acid sequence of the cellular protein which interacts with the RapR6 gene product can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, e.g., Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence

obtained may be used as a guide for the generation of oligonucleotide mixtures that can be  
5 used to screen for gene sequences encoding such cellular proteins. Screening made be  
accomplished, for example, by standard hybridization or PCR techniques. Techniques for  
the generation of oligonucleotide mixtures and the screening are well-known. (See, *e.g.*,  
Ausubel, *supra.*, and PCR Protocols: A Guide to Methods and Applications, 1990, Innis,  
M. *et al.*, eds. Academic Press, Inc., New York).

10 Additionally, methods may be employed which result in the simultaneous  
identification of genes which encode the cellular protein interacting with the RapR6 protein.  
These methods include, for example, probing expression libraries with labeled RapR6  
protein, using RapR6 protein in a manner similar to the well known technique of antibody  
15 probing of  $\lambda$ gt11 libraries.

One method which detects protein interactions *in vivo*, the two-hybrid system, is  
described in detail for illustration only and not by way of limitation. One version of this  
system has been described (Chien *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582)  
and is commercially available from Clontech (Palo Alto, CA).

20 Briefly, utilizing such a system, plasmids are constructed that encode two hybrid  
proteins: one consists of the DNA-binding domain of a transcription activator protein fused  
to the RapR6 gene product and the other consists of the transcription activator protein's  
activation domain fused to an unknown protein that is encoded by a cDNA which has been  
25 recombined into this plasmid as part of a cDNA library. The DNA-binding domain fusion  
plasmid and the cDNA library are transformed into a strain of the yeast *Saccharomyces  
cerevisiae* that contains a reporter gene (*e.g.*, HBS or *lacZ*) whose regulatory region  
contains the transcription activator's binding site. Either hybrid protein alone cannot  
activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because  
30 it does not provide activation function and the activation domain hybrid cannot because it  
cannot localize to the activator's binding sites. Interaction of the two hybrid proteins  
reconstitutes the functional activator protein and results in expression of the reporter gene,  
which is detected by an assay for the reporter gene product.

35 The two-hybrid system or related methodology may be used to screen activation  
domain libraries for proteins that interact with the "bait" gene product. By way of example,  
and not by way of limitation, RapR6 gene products may be used as the bait gene product.  
Total genomic or cDNA sequences are fused to the DNA encoding an activation domain.



This library and a plasmid encoding a hybrid of a bait RapR6 gene product fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, and not by way of limitation, a bait RapR6 gene sequence, such as the coding sequence of a RapR6 gene can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait RapR6 gene product are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transformed along with the bait RapR6 gene-GAL4 fusion plasmid into a yeast strain which contains a *lacZ* gene driven by a promoter which contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait RapR6 gene product will reconstitute an active GAL4 protein and thereby drive expression of the *HIS3* gene. Colonies which express *HIS3* can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to produce and isolate the bait RapR6 gene-interacting protein using techniques routinely practiced in the art.

The interaction between a RapR6 gene and its regulators may be determined by a standard method known in the art.

In one embodiment, the invention provides Cyclin D1 and *cdc2* as a molecule involved in the RapR6 related rapamycin regulatory pathway (see FIGS. 8D-8E). Elevated expression of cyclin D1 and/or *cdc2* have been shown in various cancers (see, e.g., Shintani et al., 2002, *Oral Oncol.* 38:235-43). *Cdc2* protein kinase (also termed p34 protein kinase) which is activated by forming a complex with cyclin B and is required for the G2/M transition, i.e., the transition from G2 phase to mitosis, of the cell cycle (see, e.g., Smits et al., 2001, *Biochim Biophys Acta* 1519:1-12; Draetta et al., 1988, *Cell* 54:17-26; Lee et al., 1987, *Nature* 327:31-35; Pines et al., 1989, *Cell* 58:833-846). Reduction in *cdc2* expression and/or activity has been shown to lead to G2/M arrest. An analysis of the murine *cdc2* gene

has shown that *cdc2* gene, while lacking a TATA box in its promoter region, utilizes multiple transcriptional start sites, including transcription factor binding sites for PEA3, CREB, C/EBP, E box factor, YY1, ATF-like, Spl, and E2F (Jun et al., 1998, Mol. Cells 8:731-40). Therefore the expression of *cdc2* gene may be controlled by a variety of different factors. For example, it has been reported that a checkpoint protein Chfr delays entry into mitosis via negatively regulating phosphorylation of *cdc2* (Kang et al., 2002, J. Cell Biol. 156:249-60). It has also been reported that LATS1, a mammalian tumor suppressor gene, inhibits cell proliferation by reducing *cdc2* kinase activity and causing G2/M blockade (Xia et al., 2002, Oncogene 21:1233-41). p53 has also been shown to negatively regulate *cdc2* gene expression via binding to an inverted CCAAT sequence in the presence of the transcription factor NF-Y (Yun et al., 1999, J. Biol. Chem. 274:29677-82). Other cellular proteins that regulate *cdc2* gene expression include the upstream stimulatory factors, a subset of Helix-Loop-Helix family of transcription factors, which binds to the CAGGTGGC sequence contained in an E-box (North et al., 1999, Oncogene 18:1945-55). Extracellular factors may also affect *cdc2* expression and/or activity and lead to G2/M arrest. For example, it has been reported that the inhibition of phosphorylation of *cdc2* by a reovirus leads to inhibition of cellular proliferation by inducing G2/M cell cycle arrest (Poggioli et al., 2001, J Virol 75(16):7429-34). It has also been reported that the anti-cancer activity of a synthetic quinoxaline phenoxypropionic acid derivative, 2-[4-(7-chloro-2-quinoxalinyloxy) phenoxy]propionic acid, is achieved by inducing G2/M arrest via inactivation of *cdc2* kinase activity (Ding et al., 2001, Clin Cancer Res 7:3336-42).

Cyclin D1 is one of G1 cyclins. Suppression of cyclin D1 was shown to inhibit cell cycle at G0/G1. It is also reported that the all-trans-retinoic acid triggered G1 arrest is at least partly through proteasome-dependent degradation of cyclin D1 (Dragnev et al., 2001, Annals of the New York Academy of Sciences 952:13-22).

#### 5.4.2. METHODS OF SCREENING FOR AGENTS

The invention provides methods for screening for agents that regulate RapR6 expression or modulate interaction of RapR6 with other proteins or molecules.

##### 5.4.2.1. SCREENING ASSAYS

The following assays are designed to identify compounds that bind to RapR6 gene  
5 or gene products, bind to other cellular proteins that interact with a RapR6 gene product,  
bind to cellular constituents, e.g., proteins, that are affected by a RapR6 gene product, or  
bind to compounds that interfere with the interaction of the RapR6 gene or gene product  
with other cellular proteins and to compounds which modulate the activity of RapR6 gene  
(i.e., modulate the level of RapR6 gene expression and/or modulate the level of RapR6 gene  
10 product activity). Assays may additionally be utilized which identify compounds which  
bind to RapR6 gene regulatory sequences (e.g., promoter sequences), see e.g., Platt, K.A.,  
1994, J. Biol. Chem. 269:28558-28562, which is incorporated herein by reference in its  
entirety, which may modulate the level of RapR6 gene expression. Compounds may  
include, but are not limited to, small organic molecules which are able to affect expression  
15 of the RapR6 gene or some other gene involved in the rapamycin resistance regulatory  
pathways, or other cellular proteins. For example, the invention provides Cyclin D1 and  
cdc2 as molecules involved in the RapR6 related rapamyin regulatory pathway (see FIGS.  
8D-8E). Methods for the identification of such cellular proteins are described, above, in  
Section 5.4.1. Such cellular proteins may be involved in the control and/or regulation of  
20 rapamycin resistance and/or tumorigenesis. Further, among these compounds are  
compounds which affect the level of RapR6 gene expression and/or RapR6 gene product  
activity and which can be used in the regulation of rapamycin resistance and/or  
tumorigenesis.

25 Compounds may include, but are not limited to, peptides such as, for example,  
soluble peptides, including but not limited to, Ig-tailed fusion peptides, and members of  
random peptide libraries; (see, e.g., Lam, K.S. *et al.*, 1991, Nature 354:82-84; Houghten, R.  
*et al.*, 1991, Nature 354:84-86), and combinatorial chemistry-derived molecular library  
made of D- and/or L- configuration amino acids, phosphopeptides (including, but not  
30 limited to members of random or partially degenerate, directed phosphopeptide libraries;  
see, e.g., Songyang, Z. *et al.*, 1993, Cell 72:767-778), antibodies (including, but not limited  
to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies,  
and FAb, F(ab')<sub>2</sub> and FAb expression library fragments, and epitope-binding fragments  
thereof), and small organic or inorganic molecules.

35 Compounds identified via assays such as those described herein may be useful, for  
example, in regulating the biological function of the RapR6 gene product, and for

ameliorating rapamycin resistance and/or inhibiting the growth of cancer cells. Assays for  
5 testing the effectiveness of compounds are discussed, below, in Section 5.4.2.2.

*In vitro* systems may be designed to identify compounds capable of binding the  
RapR6 gene products of the invention. Compounds identified may be useful, for example,  
in modulating the activity of wild type and/or mutant RapR6 gene products, may be useful  
10 in elaborating the biological function of the RapR6 gene product, may be utilized in screens  
for identifying compounds that disrupt normal RapR6 gene product interactions, or may in  
themselves disrupt such interactions.

The principle of the assays used to identify compounds that bind to the RapR6 gene  
product involves preparing a reaction mixture of the RapR6 gene product and the test  
15 compound under conditions and for a time sufficient to allow the two components to  
interact and bind, thus forming a complex which can be removed and/or detected in the  
reaction mixture. These assays can be conducted in a variety of ways. For example, one  
method to conduct such an assay would involve anchoring RapR6 gene product or the test  
substance onto a solid phase and detecting RapR6 gene product/test compound complexes  
20 anchored on the solid phase at the end of the reaction. In one embodiment of such a  
method, the RapR6 gene product may be anchored onto a solid surface, and the test  
compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilized as the solid phase. The  
25 anchored component may be immobilized by non-covalent or covalent attachments. Non-  
covalent attachment may be accomplished by simply coating the solid surface with a  
solution of the protein and drying. Alternatively, an immobilized antibody, preferably a  
monoclonal antibody, specific for the protein to be immobilized may be used to anchor the  
protein to the solid surface. The surfaces may be prepared in advance and stored.

30 In order to conduct the assay, the nonimmobilized component is added to the coated  
surface containing the anchored component. After the reaction is complete, unreacted  
components are removed (e.g., by washing) under conditions such that any complexes  
formed will remain immobilized on the solid surface. The detection of complexes anchored  
on the solid surface can be accomplished in a number of ways. Where the previously  
35 nonimmobilized component is pre-labeled, the detection of label immobilized on the  
surface indicates that complexes were formed. Where the previously nonimmobilized  
component is not pre-labeled, an indirect label can be used to detect complexes anchored on

the surface; *e.g.*, using a labeled antibody specific for the previously nonimmobilized  
5 component (the antibody, in turn, may be directly labeled or indirectly labeled with a  
labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products  
separated from unreacted components, and complexes detected; *e.g.*, using an immobilized  
10 antibody specific for RapR6 gene product or the test compound to anchor any complexes  
formed in solution, and a labeled antibody specific for the other component of the possible  
complex to detect anchored complexes.

The RapR6 gene or gene products of the invention may, *in vivo*, interact with one or  
more intracellular or extracellular molecules, such as proteins. Such molecules may  
15 include, but are not limited to, nucleic acid molecules and those proteins identified via  
methods such as those described, above, in Section 5.4.1. For purposes of this discussion,  
such molecules are referred to herein as "binding partners". Compounds that disrupt RapR6  
gene product binding may be useful in regulating the activity of the RapR6 gene product,  
especially mutant RapR6 gene products. Compounds that disrupt RapR6 gene binding may  
20 be useful in regulating the expression of the RapR6 gene, such as by regulating the binding  
of a regulator of RapR6 gene. Such compounds may include, but are not limited to  
molecules such as peptides, and the like, as described, for example, in Section 5.4.2.1.  
above, which would be capable of gaining access to the RapR6 gene product.

25 The basic principle of the assay systems used to identify compounds that interfere  
with the interaction between the RapR6 gene product and its intracellular or extracellular  
binding partner or partners involves preparing a reaction mixture containing the RapR6  
gene product, and the binding partner under conditions and for a time sufficient to allow the  
two to interact and bind, thus forming a complex. In order to test a compound for inhibitory  
30 activity, the reaction mixture is prepared in the presence and absence of the test compound.  
The test compound may be initially included in the reaction mixture, or may be added at a  
time subsequent to the addition of RapR6 gene product and its binding partner. Control  
reaction mixtures are incubated without the test compound or with a placebo. The  
formation of any complexes between the RapR6 gene protein and the binding partner is  
35 then detected. The formation of a complex in the control reaction, but not in the reaction  
mixture containing the test compound, indicates that the compound interferes with the  
interaction of the RapR6 gene protein and the interactive binding partner. Additionally,

complex formation within reaction mixtures containing the test compound and normal  
5 RapR6 gene protein may also be compared to complex formation within reaction mixtures  
containing the test compound and a mutant RapR6 gene protein. This comparison may be  
important in those cases wherein it is desirable to identify compounds that disrupt  
interactions of mutant but not normal RapR6 gene proteins.

10 The assay for compounds that interfere with the interaction of the RapR6 gene  
products and binding partners can be conducted in a heterogeneous or homogeneous format.  
Heterogeneous assays involve anchoring either the RapR6 gene product or the binding  
partner onto a solid phase and detecting complexes anchored on the solid phase at the end  
of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase.  
15 In either approach, the order of addition of reactants can be varied to obtain different  
information about the compounds being tested. For example, test compounds that interfere  
with the interaction between the RapR6 gene products and the binding partners, *e.g.*, by  
competition, can be identified by conducting the reaction in the presence of the test  
substance; *i.e.*, by adding the test substance to the reaction mixture prior to or  
20 simultaneously with the RapR6 gene protein and interactive binding partner. Alternatively,  
test compounds that disrupt preformed complexes, *e.g.* compounds with higher binding  
constants that displace one of the components from the complex, can be tested by adding  
the test compound to the reaction mixture after complexes have been formed. The various  
formats are described briefly below.

25 In a heterogeneous assay system, either the RapR6 gene product or the interactive  
binding partner, is anchored onto a solid surface, while the non-anchored species is labeled,  
either directly or indirectly. In practice, microtiter plates are conveniently utilized. The  
anchored species may be immobilized by non-covalent or covalent attachments. Non-  
covalent attachment may be accomplished simply by coating the solid surface with a  
30 solution of the RapR6 gene product or binding partner and drying. Alternatively, an  
immobilized antibody specific for the species to be anchored may be used to anchor the  
species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to  
35 the coated surface with or without the test compound. After the reaction is complete,  
unreacted components are removed (*e.g.*, by washing) and any complexes formed will  
remain immobilized on the solid surface. The detection of complexes anchored on the solid

surface can be accomplished in a number of ways. Where the non-immobilized species is  
5 pre-labeled, the detection of label immobilized on the surface indicates that complexes were  
formed. Where the non-immobilized species is not pre-labeled, an indirect label can be  
used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for  
the initially non-immobilized species (the antibody, in turn, may be directly labeled or  
indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of  
10 reaction components, test compounds which inhibit complex formation or which disrupt  
preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or  
absence of the test compound, the reaction products separated from unreacted components,  
15 and complexes detected; *e.g.*, using an immobilized antibody specific for one of the binding  
components to anchor any complexes formed in solution, and a labeled antibody specific  
for the other partner to detect anchored complexes. Again, depending upon the order of  
addition of reactants to the liquid phase, test compounds which inhibit complex or which  
disrupt preformed complexes can be identified.

20 In an alternate embodiment of the invention, a homogeneous assay can be used. In  
this approach, a preformed complex of the RapR6 gene protein and the interactive binding  
partner is prepared in which either the RapR6 gene product or its binding partners is  
labeled, but the signal generated by the label is quenched due to complex formation (see,  
*e.g.*, U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for  
25 immunoassays). The addition of a test substance that competes with and displaces one of  
the species from the preformed complex will result in the generation of a signal above  
background. In this way, test substances which disrupt RapR6 gene protein/binding partner  
interaction can be identified.

30 In a particular embodiment, the RapR6 gene product can be prepared for  
immobilization using recombinant DNA techniques described in Section 5.2. above. For  
example, the RapR6 coding region can be fused to a glutathione-S-transferase (GST) gene  
using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is  
maintained in the resulting fusion protein. The interactive binding partner can be purified  
35 and used to raise a monoclonal antibody, using methods routinely practiced in the art and  
described above, in Section 5.3. This antibody can be labeled with the radioactive isotope  
<sup>125</sup>I, for example, by methods routinely practiced in the art. In a heterogeneous assay, *e.g.*,

the GST-RapR6 fusion protein can be anchored to glutathione-agarose beads. The  
5 interactive binding partner can then be added in the presence or absence of the test  
compound in a manner that allows interaction and binding to occur. At the end of the  
reaction period, unbound material can be washed away, and the labeled monoclonal  
antibody can be added to the system and allowed to bind to the complexed components.  
The interaction between the RapR6 gene protein and the interactive binding partner can be  
10 detected by measuring the amount of radioactivity that remains associated with the  
glutathione-agarose beads. A successful inhibition of the interaction by the test compound  
will result in a decrease in measured radioactivity.

Alternatively, the GST-RapR6 gene fusion protein and the interactive binding  
15 partner can be mixed together in liquid in the absence of the solid glutathione-agarose  
beads. The test compound can be added either during or after the species are allowed to  
interact. This mixture can then be added to the glutathione-agarose beads and unbound  
material is washed away. Again the extent of inhibition of the RapR6 gene product/binding  
partner interaction can be detected by adding the labeled antibody and measuring the  
20 radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed  
using peptide fragments that correspond to the binding domains of the RapR6 protein  
and/or the interactive binding partner (in cases where the binding partner is a protein), in  
place of one or both of the full length proteins. Any number of methods routinely practiced  
25 in the art can be used to identify and isolate the binding sites. These methods include, but  
are not limited to, mutagenesis of the gene encoding one of the proteins and screening for  
disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the  
gene encoding the second species in the complex can then be selected. Sequence analysis  
of the genes encoding the respective proteins will reveal the mutations that correspond to  
30 the region of the protein involved in interactive binding. Alternatively, one protein can be  
anchored to a solid surface using methods described in this Section above, and allowed to  
interact with and bind to its labeled binding partner, which has been treated with a  
proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the  
binding domain may remain associated with the solid material, which can be isolated and  
35 identified by amino acid sequencing. Also, once the gene coding for the binding partner is  
obtained, short gene segments can be engineered to express peptide fragments of the  
protein, which can then be tested for binding activity and purified or synthesized.



For example, and not by way of limitation, a RapR6 gene product can be anchored  
5 to a solid material as described, above, in this Section by making a GST-RapR6 fusion  
protein and allowing it to bind to glutathione agarose beads. The interactive binding partner  
can be labeled with a radioactive isotope, such as  $^{35}\text{S}$ , and cleaved with a proteolytic  
enzyme such as trypsin. Cleavage products can then be added to the anchored GST-RapR6  
10 fusion protein and allowed to bind. After washing away unbound peptides, labeled bound  
material, representing the binding partner binding domain, can be eluted, purified, and  
analyzed for amino acid sequence by well-known methods. Peptides so identified can be  
produced synthetically or fused to appropriate facilitative proteins using recombinant DNA  
technology.

15 5.4.2.2. SCREENING COMPOUNDS THAT REGULATE RAPAMYCIN RESISTANCE  
AND/OR TUMORGENESIS

Any agents that regulate the expression of RapR6 gene and/or the interaction of  
RapR6 protein with its binding partners, e.g., compounds that are identified in Section  
5.4.2.1., antibodies to RapR6 protein, and so on, can be further screened for its ability to  
20 regulate rapamycin resistance and/or tumorigenesis in cells. Any suitable proliferation or  
growth inhibition assays known in the art can be used for this purpose. In one embodiment,  
a candidate agent and rapamycin are applied to a cells of a cell line, such as but not limited  
to, a rapamycin resistance cell line, and a change in growth inhibitory effect is determined.  
Preferably, changes in growth inhibitory effect are determined using different  
25 concentrations of the candidate agent in conjunction with different concentrations of  
rapamycin such that one or more combinations of concentrations of the candidate agent and  
rapamycin which cause 50% inhibition, i.e., the  $\text{IC}_{50}$ , are determined.

In a preferred embodiment, an MTT proliferation assay (see, e.g., van de  
Loosdrechet, et al., 1994, J. Immunol. Methods 174: 311-320; Ohno et al., 1991, J.  
30 Immunol. Methods 145:199-203; Ferrari et al., 1990, J. Immunol. Methods 131: 165-172;  
Alley et al., 1988, Cancer Res. 48: 589-601; Carmichael et al., 1987, Cancer Res. 47:936-  
942; Gerlier et al., 1986, J. Immunol. Methods 65:55-63; Mosmann, 1983, J.  
Immunological Methods 65:55-63) is used to screen for a candidate agent in conjunction  
with rapamycin to inhibit the growth of rapamycin resistant cells. The cells are treated with  
35 chosen concentrations of the candidate agent and rapamycin for 4 to 72 hours. The cells are  
then incubated with a suitable amount of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) for 1-8 hours such that viable cells convert MTT into  
5 an intracellular deposit of insoluble formazan. After removing the excess MTT contained  
in the supernatant, a suitable MTT solvent, e.g., a DMSO solution, is added to dissolve the  
formazan. The concentration of MTT, which is proportional to the number of viable cells,  
is then measured by determining the optical density at 570 nm. A plurality of different  
10 concentrations of the candidate agent can be assayed to allow the determination of the  
concentrations of the candidate agent and rapamycin which causes 50% inhibition.

#### 5.4.2.3. COMPOUNDS IDENTIFIED

The compounds identified in the screen include compounds that demonstrate the  
ability to selectively modulate the expression of RapR6 and regulate rapamycin resistance  
15 and/or tumorigenesis. These compounds include but are not limited to nucleic acid encoding  
RapR6 and homologues, analogues, and deletions thereof, as well as antisense, ribozyme,  
triple helix, dsRNAs, antibody, and polypeptide molecules and small organic or inorganic  
molecules.

20 The compounds identified in the screen also include compounds that modulate  
interaction of RapR6 with other proteins or molecules. In one embodiment, the compounds  
identified in the screen are compounds that modulate the interaction of a RapR6 protein  
with its interaction partner. In another embodiment, the compounds identified in the screen  
are compounds that modulate the interaction of a RapR6 protein with proteins or molecules  
25 that bind a signal domain or a WD40 domain of the RapR6 protein. In another  
embodiment, the compounds identified in the screen are compounds that modulate the  
interaction of a RapR6 protein with proteins or molecules that bind a transmembrane  
domain of the RapR6 protein. In another embodiment, the compounds identified in the  
screen are compounds that modulate the interaction of RapR6 gene with a transcription  
30 regulator.

#### 5.4.3. DIAGNOSTICS

A variety of methods can be employed for the diagnostic and prognostic evaluation  
of rapamycin resistance and/or tumorigenesis resulting from defective regulation of RapR6,  
35 and for the identification of subjects having a predisposition to rapamycin resistance and/or  
tumorigenesis.

The invention provides methods for diagnosing in a mammal a cancer which is a  
5 result of defective regulation of a RapR6 gene or a predisposition to such a cancer. In one  
embodiment, the method comprises determining an expression level of the RapR6 gene in  
cells of the mammal, in which an expression level deviated from a predetermined threshold  
level indicates that the mammal has or is predisposed of the cancer. Preferably, the  
predetermined threshold level is at least 2-fold, 4-fold, 8-fold, or 10-fold of the normal  
10 expression level of the RapR6 gene. In another embodiment, the invention provides a  
method for diagnosing in a mammal a cancer which is a result of defective regulation of a  
RapR6 gene or a predisposition to such a cancer comprising determining a level of  
abundance of a protein encoded by the RapR6 gene in cells of the mammal, in which a level  
of abundance of the protein deviated from a predetermined threshold level indicates that the  
15 mammal has or is predisposed of the cancer. In still another embodiment, the invention  
provides a method for diagnosing the cancer comprising determining a level of activity of a  
protein encoded by the RapR6 gene in cells of the mammal, in which an activity level  
deviated from a predetermined threshold level indicates that the mammal has or is  
predisposed of the cancer. As used herein, activities of a RapR6 protein include but not  
20 limited to its binding properties, e.g., binding specificity to a binding partner. Preferably,  
the predetermined threshold level of abundance or activity is at least 2-fold, 4-fold, 8-fold,  
or 10-fold of the normal level of abundance or activity of the RapR6 protein.

The invention also provides methods for evaluating rapamycin resistance in a cell.  
25 In one embodiment, the method comprises determining an expression level of a RapR6  
gene in the cell, in which an expression level deviated from a predetermined threshold level  
indicates that the cell is rapamycin resistant. Preferably, the predetermined threshold level  
is at least 2-fold, 4-fold, 8-fold, or 10-fold of the normal expression level of the RapR6  
gene. In another embodiment, the invention provides a method for evaluating rapamycin  
30 resistance in a cell comprising determining a level of abundance of a protein encoded by a  
RapR6 gene in the cell, in which a level of abundance of the protein deviated from a  
predetermined threshold level indicates that the cell is rapamycin resistant. In still another  
embodiment, the invention provides a method for evaluating rapamycin resistance in a cell  
comprising determining a level of activity of a protein encoded by the RapR6 gene in cells  
35 of the mammal, in which an activity level deviated from a predetermined threshold level  
indicates that the cell is rapamycin resistant. Preferably, the predetermined threshold level  
of abundance or activity is at least 2-fold, 4-fold, 8-fold, or 10-fold of the normal level of  
abundance or activity of the RapR6 protein.

Such methods may, for example, utilize reagents such as the RapR6 gene nucleotide  
5 sequences described in Sections 5.1, and antibodies directed against RapR6 gene products,  
including peptide fragments thereof, as described, above, in Section 5.3. Specifically, such  
reagents may be used, for example, for: (1) the detection of the presence of RapR6 gene  
mutations, or the detection of either over- or under-expression of RapR6 gene mRNA  
relative to the normal expression level; and (2) the detection of either an over- or an under-  
10 abundance of RapR6 gene product relative to the normal RapR6 protein level.

The methods described herein may be performed, for example, by utilizing pre-  
packaged diagnostic kits comprising at least one specific RapR6 gene nucleic acid or anti-  
RapR6 gene antibody reagent described herein, which may be conveniently used, *e.g.*, in  
15 clinical settings, to diagnose patients exhibiting RapR6 related disorder or abnormalities.

For the detection of RapR6 mutations, any nucleated cell can be used as a starting  
source for genomic nucleic acid. For the detection of RapR6 gene expression or RapR6  
gene products, any cell type or tissue in which the RapR6 gene is expressed, such as, for  
example, hypothalamus cells, may be utilized.  
20

Nucleic acid-based detection techniques are described, below, in Section 5.4.3.1.  
Peptide detection techniques are described, below, in Section 5.4.3.2.

#### 5.4.3.1. DETECTION OF EXPRESSION OF RAPR6 GENE

25 The expression of RapR6 gene in cells or tissues, *e.g.*, the cellular level of RapR6  
transcripts and/or the presence or absence of mutations, can be detected by utilizing a  
number of techniques. Nucleic acid from any nucleated cell can be used as the starting  
point for such assay techniques, and may be isolated according to standard nucleic acid  
preparation procedures which are well known to those of skill in the art. For example, the  
30 expression level of the RapR6 gene can determined by measuring the expression level of  
the RapR6 gene using one or more polynucleotide probes, each of which comprises a  
nucleotide sequence in the RapR6 gene. In one embodiment, the one or more  
polynucleotide probes comprise at least one polynucleotide probe comprising a nucleotide  
sequence within one of exons 1-6 of the RapR6 gene. In another embodiment, the one or  
35 more polynucleotide probes comprise at least one polynucleotide probe comprising a  
nucleotide sequence within an intron of the RapR6 gene. In particularly preferred  
embodiments of the invention, the methods is used to diagnose the cancer in a human.

DNA may be used in hybridization or amplification assays of biological samples to  
5 detect abnormalities involving RapR6 gene structure, including point mutations, insertions,  
deletions and chromosomal rearrangements. Such assays may include, but are not limited  
to, Southern analyses, single stranded conformational polymorphism analyses (SSCP),  
DNA microarray analyses, and PCR analyses.

10 Such diagnostic methods for the detection of RapR6 gene-specific mutations can  
involve for example, contacting and incubating nucleic acids including recombinant DNA  
molecules, cloned genes or degenerate variants thereof, obtained from a sample, *e.g.*,  
derived from a patient sample or other appropriate cellular source, with one or more labeled  
nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate  
15 variants thereof, as described in Section 5.1, under conditions favorable for the specific  
annealing of these reagents to their complementary sequences within the RapR6 gene.  
Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After  
incubation, all non-annealed nucleic acids are removed from the nucleic acid:RapR6  
molecule hybrid. The presence of nucleic acids which have hybridized, if any such  
20 molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the  
cell type or tissue of interest can be immobilized, for example, to a solid support such as a  
membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In  
this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described  
in Section 5.1 are easily removed. Detection of the remaining, annealed, labeled RapR6  
25 nucleic acid reagents is accomplished using standard techniques well-known to those in the  
art. The RapR6 gene sequences to which the nucleic acid reagents have annealed can be  
compared to the annealing pattern expected from a normal RapR6 gene sequence in order to  
determine whether a RapR6 gene mutation is present.

Alternative diagnostic methods for the detection of RapR6 gene specific nucleic acid  
30 molecules, in patient samples or other appropriate cell sources, may involve their  
amplification, *e.g.*, by PCR (the experimental embodiment set forth in Mullis, K.B., 1987,  
U.S. Patent No. 4,683,202), followed by the detection of the amplified molecules using  
techniques well known to those of skill in the art. The resulting amplified sequences can be  
compared to those which would be expected if the nucleic acid being amplified contained  
35 only normal copies of the RapR6 gene in order to determine whether a RapR6 gene  
mutation exists.

Among the RapR6 nucleic acid sequences which are preferred for such  
5 hybridization and/or PCR analyses are those which will detect the presence of the RapR6  
gene splice site mutation.

Additionally, well-known genotyping techniques can be performed to identify  
individuals carrying RapR6 gene mutations. Such techniques include, for example, the use  
10 of restriction fragment length polymorphisms (RFLPs), which involve sequence variations  
in one of the recognition sites for the specific restriction enzyme used.

Additionally, improved methods for analyzing DNA polymorphisms which can be  
utilized for the identification of RapR6 gene mutations have been described which  
capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences  
15 between the restriction enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217, which  
is incorporated herein by reference in its entirety) describes a DNA marker based on length  
polymorphisms in blocks of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> short tandem repeats. The average  
separation of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> blocks is estimated to be 30,000-60,000 bp. Markers  
which are so closely spaced exhibit a high frequency co-inheritance, and are extremely  
20 useful in the identification of genetic mutations, such as, for example, mutations within the  
RapR6 gene, and the diagnosis of diseases and disorders related to RapR6 mutations.

Also, Caskey *et al.* (U.S. Pat.No. 5,364,759, which is incorporated herein by  
reference in its entirety) describe a DNA profiling assay for detecting short tri and tetra  
25 nucleotide repeat sequences. The process includes extracting the DNA of interest, such as  
the RapR6 gene, amplifying the extracted DNA, and labelling the repeat sequences to form  
a genotypic map of the individual's DNA.

The level of RapR6 gene expression can also be assayed. For example, RNA from a  
cell type or tissue known, or suspected, to express the RapR6 gene, such as MDCK cells or  
30 from a cell line which exhibits rapamycin resistance, may be isolated and tested utilizing  
hybridization or PCR techniques such as are described, above. The isolated cells can be  
derived from cell culture or from a patient. The analysis of cells taken from culture may be  
a necessary step in the assessment of cells to be used as part of a cell-based gene therapy  
technique or, alternatively, to test the effect of compounds on the expression of the RapR6  
35 gene. Such analyses may reveal both quantitative and qualitative aspects of the expression  
pattern of the RapR6 gene, including activation or inactivation of RapR6 gene expression.

In one embodiment of such a detection scheme, a cDNA molecule is synthesized  
5 from an RNA molecule of interest (*e.g.*, by reverse transcription of the RNA molecule into  
cDNA). A sequence within the cDNA is then used as the template for a nucleic acid  
amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid  
reagents used as synthesis initiation reagents (*e.g.*, primers) in the reverse transcription and  
nucleic acid amplification steps of this method are chosen from among the RapR6 gene  
10 nucleic acid reagents described in Section 5.1. The preferred lengths of such nucleic acid  
reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic  
acid amplification may be performed using radioactively or non-radioactively labeled  
nucleotides. Alternatively, enough amplified product may be made such that the product  
may be visualized by utilizing any suitable nucleic acid staining method, *e.g.*, by standard  
15 ethidium bromide staining.

Additionally, it is possible to perform such RapR6 gene expression assays “*in situ*”,  
*i.e.*, directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from  
biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid  
20 reagents such as those described in Section 5.1 may be used as probes and/or primers for  
such *in situ* procedures (see, for example, Nuovo, G.J., 1992, “PCR *In Situ* Hybridization:  
Protocols And Applications”, Raven Press, NY).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained,  
standard Northern analysis can be performed to determine the level of mRNA expression of  
25 the RapR6 gene.

The expression of RapR6 gene in cells or tissues, *e.g.*, the cellular level of RapR6  
transcripts and/or the presence or absence of mutations, can also be evaluated using DNA  
microarray technologies. In such technologies, one or more polynucleotide probes each  
30 comprising a sequence of the RapR6 gene are used to monitor the expression of the RapR6  
gene. The present invention therefore provides DNA microarrays comprising  
polynucleotide probes comprising sequences of the RapR6 gene.

Any formats of DNA microarray technologies can be used in conjunction with the  
present invention. In one embodiment, spotted cDNA arrays are prepared by depositing  
35 PCR products of cDNA fragments, *e.g.*, full length cDNAs, ESTs, etc., of the RapR6 gene  
onto a suitable surface (see, *e.g.*, DeRisi *et al.*, 1996, *Nature Genetics* 14:457-460; Shalon  
*et al.*, 1996, *Genome Res.* 6:689-645; Schena *et al.*, 1995, *Proc. Natl. Acad. Sci. U.S.A.*

93:10539-11286; and Duggan *et al.*, *Nature Genetics* Supplement 21:10-14). In another  
5 embodiment, high-density oligonucleotide arrays containing oligonucleotides  
complementary to sequences of RapR6 gene are synthesized *in situ* on the surface by  
photolithographic techniques (see, e.g., Fodor *et al.*, 1991, *Science* 251:767-773; Pease *et al.*,  
1994, *Proc. Natl. Acad. Sci. U.S.A.* 91:5022-5026; Lockhart *et al.*, 1996, *Nature*  
*Biotechnology* 14:1675; McGall *et al.*, 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93:13555-13560;  
10 U.S. Patent Nos. 5,578,832; 5,556,752; 5,510,270; 5,858,659; and 6,040,138). This format  
of microarray technology is particularly useful for detection of single nucleotide  
polymorphisms (SNPs) (see, e.g., Hacia *et al.*, 1999, *Nat Genet.* 22:164-7; Wang *et al.*,  
1998, *Science* 280:1077-82). In yet another embodiment, high-density oligonucleotide  
arrays containing oligonucleotides complementary to sequences of RapR6 gene are  
15 synthesized *in situ* on the surface by inkjet technologies (see, e.g., Blanchard, International  
Patent Publication WO 98/41531, published September 24, 1998; Blanchard *et al.*, 1996,  
*Biosensors and Bioelectronics* 11:687-690; Blanchard, 1998, in *Synthetic DNA Arrays in*  
*Genetic Engineering*, Vol. 20, J.K. Setlow, Ed., Plenum Press, New York at pages 111-  
123). In still another embodiment, DNA microarrays that allow electronic stringency  
20 control can be used in conjunction with polynucleotide probes comprising sequences of the  
RapR6 gene (see, e.g., U.S. Patent No. 5,849,486).

#### 5.4.3.2. DETECTION OF RAPR6 GENE PRODUCTS

Antibodies directed against wild type or mutant RapR6 gene products or conserved  
25 variants or peptide fragments thereof, which are discussed, above, in Section 5.3, may also  
be used as diagnostics and prognostics of rapamycin resistance and/or tumorigenesis, as  
described herein. Such diagnostic methods, may be used to detect abnormalities in the level  
of RapR6 gene expression, or abnormalities in the structure and/or temporal, tissue, cellular,  
or subcellular location of RapR6 gene product. In exemplary embodiments of the  
30 invention, the protein detected is a human RapR6 protein as depicted in SEQ ID NO:11, or  
a murine RapR6 protein as depicted in SEQ ID NO:3.

Because evidence disclosed herein indicates that the RapR6 gene product is an  
intracellular gene product, the antibodies and immunoassay methods described below have  
35 important *in vitro* applications in assessing the efficacy of treatments for disorders resulting  
from defective regulation of RapR6 gene such as infectious diseases, immunodeficiencies,  
autoimmune diseases, inflammatory diseases, and proliferative diseases. Antibodies, or



fragments of antibodies, such as those described below, may be used to screen potentially  
5 therapeutic compounds *in vitro* to determine their effects on RapR6 gene expression and  
RapR6 peptide production. The compounds which have beneficial effects on disorders  
related to defective regulation of RapR6 can be identified, and a therapeutically effective  
dose determined.

10 *In vitro* immunoassays may also be used, for example, to assess the efficacy of cell-  
based gene therapy for disorders related to defective regulation of RapR6. Antibodies  
directed against RapR6 peptides may be used *in vitro* to determine the level of RapR6 gene  
expression achieved in cells genetically engineered to produce RapR6 peptides. Given that  
evidence disclosed herein indicates that the RapR6 gene product is an intracellular gene  
15 product, such an assessment is, preferably, done using cell lysates or extracts. Such  
analysis will allow for a determination of the number of transformed cells necessary to  
achieve therapeutic efficacy *in vivo*, as well as optimization of the gene replacement  
protocol.

20 The tissue or cell type to be analyzed will generally include those which are known,  
or suspected, to express the RapR6 gene, such as, for example, hypothalamic cells. The  
protein isolation methods employed herein may, for example, be such as those described in  
Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual",  
Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is  
25 incorporated herein by reference in its entirety. The isolated cells can be derived from cell  
culture or from a patient. The analysis of cell taken from culture may be a necessary step in  
the assessment of cells to be used as part of a cell-based gene therapy technique or,  
alternatively, to test the effect of compounds on the expression of the RapR6 gene.

Preferred diagnostic methods for the detection of RapR6 gene products or conserved  
30 variants or peptide fragments thereof, may involve, for example, immunoassays wherein the  
RapR6 gene products or conserved variants or peptide fragments are detected by their  
interaction with an anti-RapR6 gene product-specific antibody.

For example, antibodies, or fragments of antibodies, such as those described, above,  
35 in Section 5.3, useful in the present invention may be used to quantitatively or qualitatively  
detect the presence of RapR6 gene products or conserved variants or peptide fragments  
thereof. This can be accomplished, for example, by immunofluorescence techniques  
employing a fluorescently labeled antibody (see below, this Section) coupled with light

microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially  
5 preferred if such RapR6 gene products are expressed on the cell surface.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of RapR6 gene products or conserved variants or peptide  
10 fragments thereof. *In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the RapR6 gene product, or conserved variants or  
15 peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays for RapR6 gene products or conserved variants or peptide fragments  
20 thereof will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying RapR6 gene products or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art.

25 The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled RapR6 protein specific  
30 antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an  
35 antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material

may have virtually any possible structural configuration so long as the coupled molecule is  
5 capable of binding to an antigen or antibody. Thus, the support configuration may be  
spherical, as in a bead, or cylindrical, as in the inside surface of a test tub, or the external  
surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.  
Preferred supports include polystyrene beads. Those skilled in the art will know many  
other suitable carriers for binding antibody or antigen, or will be able to ascertain the same  
10 by use of routine experimentation.

The binding activity of a given lot of anti-RapR6 gene product antibody may be  
determined according to well known methods. Those skilled in the art will be able to  
determine operative and optimal assay conditions for each determination by employing  
15 routine experimentation.

One of the ways in which the RapR6 gene peptide-specific antibody can be  
detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay  
(EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic  
Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, MD);  
20 Voller, A. *et al.*, 1978, J. Clin. Pathol. 31:507-520; Butler, J.E., 1981, Meth. Enzymol.  
73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, FL,;  
Ishikawa, E. *et al.*, (eds.), 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo). The enzyme  
which is bound to the antibody will react with an appropriate substrate, preferably a  
chromogenic substrate, in such a manner as to produce a chemical moiety which can be  
25 detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes  
which can be used to detectably label the antibody include, but are not limited to, malate  
dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol  
dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase,  
horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-  
30 galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase,  
glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric  
methods which employ a chromogenic substrate for the enzyme. Detection may also be  
accomplished by visual comparison of the extent of enzymatic reaction of a substrate in  
comparison with similarly prepared standards.

35  
Detection may also be accomplished using any of a variety of other immunoassays.  
For example, by radioactively labeling the antibodies or antibody fragments, it is possible to

detect RapR6 gene peptides through the use of a radioimmunoassay (RIA) (see, for  
5 example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on  
Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated  
by reference herein). The radioactive isotope can be detected by such means as the use of a  
gamma counter or a scintillation counter or by autoradiography.

10 It is also possible to label the antibody with a fluorescent compound. When the  
fluorescently labeled antibody is exposed to light of the proper wave length, its presence  
can then be detected due to fluorescence. Among the most commonly used fluorescent  
labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin,  
phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

15 The antibody can also be detectably labeled using fluorescence emitting metals such  
as <sup>152</sup>Eu, or others of the lanthanide series. These metals can be attached to the antibody  
using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or  
ethylenediaminetetraacetic acid (EDTA).

20 The antibody also can be detectably labeled by coupling it to a chemiluminescent  
compound. The presence of the chemiluminescent-tagged antibody is then determined by  
detecting the presence of luminescence that arises during the course of a chemical reaction.  
Examples of particularly useful chemiluminescent labeling compounds are luminol,  
isoluminol, therrromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

25 Likewise, a bioluminescent compound may be used to label the antibody of the  
present invention. Bioluminescence is a type of chemiluminescence found in biological  
systems in, which a catalytic protein increases the efficiency of the chemiluminescent  
reaction. The presence of a bioluminescent protein is determined by detecting the presence  
of luminescence. Important bioluminescent compounds for purposes of labeling are  
30 luciferin, luciferase and aequorin.

#### 5.4.4. METHODS OF REGULATING EXPRESSION OF RAPR6 GENE

A variety of therapeutic approaches may be used in accordance with the invention to  
modulate expression of the RapR6 gene *in vivo*. For example, antisense DNA molecules  
35 may be engineered and used to block translation of RapR6 mRNA *in vivo*. Alternatively,  
ribozyme molecules may be designed to cleave and destroy the RapR6 mRNAs *in vivo*. In  
another alternative, oligonucleotides designed to hybridize to the 5' region of the RapR6

gene (including the region upstream of the coding sequence) and form triple helix structures  
5 may be used to block or reduce transcription of the RapR6 gene. Oligonucleotides can also  
be designed to hybridize and form triple helix structures with the binding site of a negative  
regulator so as to block the binding of the negative regulator and to enhance the  
transcription of the RapR6 gene. In yet another alternative, nucleic acid encoding the full  
length wild-type RapR6 message may be introduced *in vivo* into cells which otherwise  
10 would be unable to produce the wild-type RapR6 gene product in sufficient quantities or at  
all. In yet another embodiment, a heterologous regulatory element may be inserted before  
the coding sequence of a RapR6 gene, such that it is operatively linked with and activates  
expression of the endogenous RapR6 gene.

15 In a preferred embodiment, the antisense, ribozyme, and triple helix nucleotides are  
designed to inhibit the translation or transcription of one or more of RapR6 isoforms with  
minimal effects on the expression of other genes that may share one or more sequence motif  
with a RapR6. To accomplish this, the oligonucleotides used should be designed on the  
basis of relevant sequences unique to RapR6.

20 For example, and not by way of limitation, the oligonucleotides should not fall  
within those region where the nucleotide sequence of RapR6 is most homologous to that of  
other genes, e.g., the WD40 domain. Instead, it is preferred that the oligonucleotides fall  
within the portion of the sequence of RapR6 that does not encode a WD40 domain, e.g.,  
amino acids 155-202, or any fragment thereof, of a human RapR6 protein, or its murine  
25 homolog. In the case of antisense molecules, it is preferred that the sequence be chosen  
from the list above. It is also preferred that the sequence be at least 18 nucleotides in length  
in order to achieve sufficiently strong annealing to the target mRNA sequence to prevent  
translation of the sequence. Izant et al., 1984, Cell, 36:1007-1015; Rosenberg *et al.*, 1985,  
Nature, 313:703-706.

30 In the case of the "hammerhead" type of ribozymes, it is also preferred that the  
target sequences of the ribozymes be chosen from the list above. Ribozymes are RNA  
molecules which possess highly specific endoribonuclease activity. Hammerhead  
ribozymes comprise a hybridizing region which is complementary in nucleotide sequence to  
35 at least part of the target RNA, and a catalytic region which is adapted to cleave the target  
RNA. The hybridizing region contains nine (9) or more nucleotides. Therefore, the  
hammerhead ribozymes of the present invention have a hybridizing region which is

complementary to the sequences listed above and is at least nine nucleotides in length. The  
5 construction and production of such ribozymes is well known in the art and is described  
more fully in Haseloff et al., 1988, Nature, 334:585-591.

The ribozymes of the present invention also include RNA endoribonucleases  
(hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena  
10 Thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively  
described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, Science, 224:574-578;  
Zaug and Cech, 1986, Science, 231:470-475; Zaug, *et al.*, 1986, Nature, 324:429-433;  
published International patent application No. WO 88/04300 by University Patents Inc.;  
Been et al., 1986, Cell, 47:207-216). The Cech endoribonucleases have an eight base pair  
15 active site which hybridizes to a target RNA sequence whereafter cleavage of the target  
RNA takes place.

In the case of oligonucleotides that hybridize to and form triple helix structures at  
the 5' terminus of the RapR6 gene and can be used to block transcription, it is preferred that  
they be complementary to those sequences in the 5' terminus of RapR6 which are not  
20 present in other RapR6 related genes. It is also preferred that the sequences not include  
those regions of the RapR6 promoter which are even slightly homologous to that of other  
RapR6 related genes. The foregoing compounds can be administered by a variety of  
methods which are known in the art including, but not limited to the use of liposomes as a  
delivery vehicle. Naked DNA or RNA molecules may also be used where they are in a  
25 form which is resistant to degradation such as by modification of the ends, by the formation  
of circular molecules, or by the use of alternate bonds including phosphothionate and  
thiophosphoryl modified bonds. In addition, the delivery of nucleic acid may be by  
facilitated transport where the nucleic acid molecules are conjugated to poly-lysine or  
transferrin. Nucleic acid may also be transported into cells by any of the various viral  
30 carriers, including but not limited to, retrovirus, vaccinia, AAV, and adenovirus.

Alternatively, a recombinant nucleic acid molecule which encodes, or is, such  
antisense, ribozyme, triple helix, or RapR6 molecule can be constructed. This nucleic acid  
molecule may be either RNA or DNA. If the nucleic acid encodes an RNA, it is preferred  
35 that the sequence be operatively attached to a regulatory element so that sufficient copies of  
the desired RNA product are produced. The regulatory element may permit either  
constitutive or regulated transcription of the sequence. *In vivo*, that is, within the cells or

cells of an organism, a transfer vector such as a bacterial plasmid or viral RNA or DNA,  
5 encoding one or more of the RNAs, may be transfected into cells *e.g.* (Llewellyn *et al.*,  
1987, *J. Mol. Biol.*, 195:115-123; Hanahan *et al.* 1983, *J. Mol. Biol.*, 166:557-580). Once  
inside the cell, the transfer vector may replicate, and be transcribed by cellular polymerases  
to produce the RNA or it may be integrated into the genome of the host cell. Alternatively,  
a transfer vector containing sequences encoding one or more of the RNAs may be  
10 transfected into cells or introduced into cells by way of micromanipulation techniques such  
as microinjection, such that the transfer vector or a part thereof becomes integrated into the  
genome of the host cell.

RNA interference (RNAi) can also be used to block expression of RapR6 (Guo *et*  
15 *al.*, 1995, *Cell* 81:611-620; Fire *et al.*, 1998, *Nature* 391:806-811; Grant, 1999, *Cell*  
96:303-306; Tabara *et al.*, 1999, *Cell* 99:123-132; Zamore *et al.*, 2000, *Cell* 101:25-33;  
Bass, 2000, *Cell* 101:235-238; Petcherski *et al.*, 2000, *Nature* 405:364-368; Elbashir *et al.*,  
*Nature* 411:494-498; Paddison *et al.*, *Proc. Natl. Acad. Sci. USA* 99:1443-1448). In one  
embodiment, double-stranded RNA molecules of 21-23 nucleotides which hybridize to a  
20 homologous region of mRNAs transcribed from the RapR6 gene are used to degrade the  
mRNAs, thereby "silence" the expression of the RapR6 gene. Preferably, the dsRNAs have  
a hybridizing region, *e.g.*, a 19-nucleotide double-stranded region, which is complementary  
to a sequence of the coding sequence of the RapR6 gene. Any siRNA targeting an  
appropriate coding sequence of a RapR6 gene, *e.g.*, a human RapR6 gene, can be used in  
25 the invention. As an exemplary embodiment, 21-nucleotide double-stranded siRNAs  
targeting the coding regions of RapR6 gene are designed according to standard selection  
rules (see, *e.g.*, Elbashir *et al.*, 2002, *Methods* 26:199-213, which is incorporated herein by  
reference in its entirety).

Any standard method for introducing nucleic acids into cells can be used. In one  
30 embodiment, gene silencing is induced by presenting the cell with the siRNA targeting the  
RapR6 gene (see, *e.g.*, Elbashir *et al.*, 2001, *Nature* 411, 494-498; Elbashir *et al.*, 2001,  
*Genes Dev.* 15, 188-200, all of which are incorporated by reference herein in their entirety).  
The siRNAs can be chemically synthesized, or derived from cleavage of double-stranded  
RNA by recombinant Dicer. Another method to introduce a double stranded DNA  
35 (dsRNA) for silencing of the RapR6 gene is shRNA, for short hairpin RNA (see, *e.g.*,  
Paddison *et al.*, 2002, *Genes Dev.* 16, 948-958; Brummelkamp *et al.*, 2002, *Science* 296,  
550-553; Sui, G. *et al.* 2002, *Proc. Natl. Acad. Sci. USA* 99, 5515-5520, all of which are

incorporated by reference herein in their entirety). In this method, an siRNA targeting  
5 RapR6 gene is expressed from a plasmid (or virus) as an inverted repeat with an intervening  
loop sequence to form a hairpin structure. The resulting RNA transcript containing the  
hairpin is subsequently processed by Dicer to produce siRNAs for silencing. Plasmid-based  
shRNAs can be expressed stably in cells, allowing long-term gene silencing in cells both *in*  
*vitro* and *in vivo* (see, McCaffrey et al. 2002, *Nature* **418**, 38-39; Xia et al., 2002, *Nat.*  
10 *Biotech.* **20**, 1006-1010; Lewis et al., 2002, *Nat. Genetics* **32**, 107-108; Robinson et al.,  
2003, *Nat. Genetics* **33**, 401-406; Tiscornia et al., 2003, *Proc. Natl. Acad. Sci. USA* **100**,  
1844-1848, all of which are incorporated by reference herein in their entirety). SiRNAs  
targeting the RapR6 gene can also be delivered to an organ or tissue in a mammal, such a  
human, *in vivo* (see, e.g., Song et al. 2003, *Nat. Medicine* **9**, 347-351; Sorensen et al., 2003,  
15 *J. Mol. Biol.* **327**, 761-766; Lewis et al., 2002, *Nat. Genetics* **32**, 107-108, all of which are  
incorporated by reference herein in their entirety). In this method, a solution of siRNA is  
injected intravenously into the mammal. The siRNA can then reach an organ or tissue of  
interest and effectively reduce the expression of the target gene in the organ or tissue of the  
mammal.

20 The expression of RapR6 genes can also be activated or enhanced. In one  
embodiment, a heterologous regulatory element may be inserted before the coding sequence  
of a RapR6 gene, such that it is operatively linked with and activates expression of the  
endogenous RapR6 gene, using techniques, such as targeted homologous recombination,  
25 which are well known to those of skill in the art, and described *e.g.*, in Chappel, U.S. Patent  
No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991; Skoultchi  
U.S. Patent No. 5,981,214; Treco *et al* U.S. Patent No. 5,968,502 and PCT publication No.  
WO 94/12650, published June 9, 1994. Alternatively, non-targeted *e.g.*, non-homologous  
recombination techniques which are well-known to those of skill in the art and described,  
30 *e.g.*, in PCT publication No. WO 99/15650, published April 1, 1999, may be used.

In another embodiment, the expression of a RapR6 gene is enhanced by blocking the  
binding of a negative regulator (*i.e.*, a repressor). Any agent that binds to such site and  
inhibit the binding of a regulator molecule, including but not limited to peptides or nucleic  
acid molecules, can be used for this purpose.

35

#### 5.4.5. GENE THERAPY BASED ON RAPR6 GENE



A variety of gene therapy approaches may be used in accordance with the invention  
5 to modulate expression of the RapR6 gene *in vivo*. In yet another alternative, nucleic acid  
encoding the full length wild-type RapR6 message may be introduced *in vivo* into cells  
which otherwise would be unable to produce the wild-type RapR6 gene product in  
sufficient quantities or at all.

10 In a specific embodiment, nucleic acids comprising a sequence encoding a RapR6 or  
functional derivative thereof, are administered to promote a RapR6 function, by way of  
gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic  
acid to a subject. In this embodiment of the invention, the nucleic acid produces its  
encoded protein that mediates a therapeutic effect by promoting a RapR6 function.

15 Any of the methods for gene therapy available in the art can be used according to  
the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993,  
Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993,  
20 Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and  
Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH  
11(5):155-215). Methods commonly known in the art of recombinant DNA technology  
which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in  
Molecular Biology, John Wiley & Sons, New York; and Kriegler, 1990, Gene Transfer and  
25 Expression, A Laboratory Manual, Stockton Press, New York.

In a preferred aspect, the therapeutic comprises a RapR6 nucleic acid that is part of  
an expression vector that expresses a RapR6 or fragment or chimeric protein thereof in a  
suitable host. In particular, such a nucleic acid has a promoter operably linked to the  
30 RapR6 coding region, said promoter being inducible or constitutive, and, optionally, tissue-  
specific. In another particular embodiment, a nucleic acid molecule is used in which the  
RapR6 coding sequences and any other desired sequences are flanked by regions that  
promote homologous recombination at a desired site in the genome, thus providing for  
intrachromosomal expression of the RapR6 nucleic acid (see e.g., Koller and Smithies,  
35 1989, Proc. Natl. Acad. Sci. U.S.A. 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-  
438).

Delivery of the nucleic acid into a patient may be either direct, in which case the  
5 patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in  
which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into  
the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene  
therapy.

10 In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it  
is expressed to produce the encoded product. This can be accomplished by any of  
numerous methods known in the art, e.g., by constructing it as part of an appropriate nucleic  
acid expression vector and administering it so that it becomes intracellular, e.g., by infection  
15 using a defective or attenuated retroviral or other viral vector (see U.S. Patent No.  
4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment  
(e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or  
transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by  
administering it in linkage to a peptide which is known to enter the nucleus, by  
20 administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g.,  
Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types  
specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand  
complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt  
endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another  
embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and  
25 expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated  
April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.);  
WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993  
(Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic  
acid can be introduced intracellularly and incorporated within host cell DNA for expression,  
30 by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. U.S.A.  
86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, a viral vector that contains the RapR6 nucleic acid is  
used. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol.  
217:581-599). These retroviral vectors have been modified to delete retroviral sequences  
35 that are not necessary for packaging of the viral genome and integration into host cell DNA.  
The RapR6 nucleic acid to be used in gene therapy is cloned into the vector, which  
facilitates delivery of the gene into a patient. More detail about retroviral vectors can be

found in Boesen et al., 1994, *Biotherapy* 6:291-302, which describes the use of a retroviral  
5 vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells  
more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in  
gene therapy are: Clowes et al., 1994, *J. Clin. Invest.* 93:644-651; Kiem et al., 1994, *Blood*  
83:1467-1473; Salmons and Gunzberg, 1993, *Human Gene Therapy* 4:129-141; and  
Grossman and Wilson, 1993, *Curr. Opin. Genet. and Devel.* 3:110-114.

10

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses  
are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses  
naturally infect respiratory epithelia where they cause a mild disease. Other targets for  
adenovirus-based delivery systems are liver, the central nervous system, endothelial cells,  
15 and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing  
cells. Kozarsky and Wilson (1993, *Current Opinion in Genetics and Development* 3:499-  
503) present a review of adenovirus-based gene therapy. Bout et al. (1994, *Human Gene*  
*Therapy* 5:3-10) demonstrated the use of adenovirus vectors to transfer genes to the  
respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene  
20 therapy can be found in Rosenfeld et al., 1991, *Science* 252:431-434; Rosenfeld et al., 1992,  
*Cell* 68:143-155; and Mastrangeli et al., 1993, *J. Clin. Invest.* 91:225-234.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy  
(Walsh et al., 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300).

25

Another approach to gene therapy involves transferring a gene to cells in tissue  
culture by such methods as electroporation, lipofection, calcium phosphate mediated  
transfection, or viral infection. Usually, the method of transfer includes the transfer of a  
selectable marker to the cells. The cells are then placed under selection to isolate those cells  
that have taken up and are expressing the transferred gene. Those cells are then delivered to  
30 a patient.

35

In this embodiment, the nucleic acid is introduced into a cell prior to administration  
in vivo of the resulting recombinant cell. Such introduction can be carried out by any  
method known in the art, including but not limited to transfection, electroporation,  
microinjection, infection with a viral or bacteriophage vector containing the nucleic acid  
35 sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene  
transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the  
introduction of foreign genes into cells (see e.g., Loeffler and Behr, 1993, *Meth. Enzymol.*

217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac.  
5 Ther. 29:69-92) and may be used in accordance with the present invention, provided that  
the necessary developmental and physiological functions of the recipient cells are not  
disrupted. The technique should provide for the stable transfer of the nucleic acid to the  
cell, so that the nucleic acid is expressible by the cell and preferably heritable and  
expressible by its cell progeny.

10

The resulting recombinant cells can be delivered to a patient by various methods  
known in the art. In a preferred embodiment, epithelial cells are injected, e.g.,  
subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin  
graft onto the patient. Recombinant blood cells (e.g., hematopoietic stem or progenitor  
15 cells) are preferably administered intravenously. The amount of cells envisioned for use  
depends on the desired effect, patient state, etc., and can be determined by one skilled  
person in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy  
encompass any desired, available cell type, and include but are not limited to epithelial  
20 cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells  
such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils,  
megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic  
stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood,  
peripheral blood, fetal liver, etc.

25

In a preferred embodiment, the cell used for gene therapy is autologous to the  
patient.

In an embodiment in which recombinant cells are used in gene therapy, a RapR6  
nucleic acid is introduced into the cells such that it is expressible by the cells or their  
30 progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a  
specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells  
which can be isolated and maintained *in vitro* can potentially be used in accordance with  
this embodiment of the present invention. Such stem cells include but are not limited to  
hematopoietic stem cells (HSC), stem cells of epithelial tissues such as the skin and the  
35 lining of the gut, embryonic heart muscle cells, liver stem cells (PCT Publication WO  
94/08598, dated April 28, 1994), and neural stem cells (Stemple and Anderson, 1992, Cell  
71:973-985).

Epithelial stem cells (ESCs) or keratinocytes can be obtained from tissues such as  
5 the skin and the lining of the gut by known procedures (Rheinwald, 1980, Meth. Cell Bio.  
21A:229). In stratified epithelial tissue such as the skin, renewal occurs by mitosis of stem  
cells within the germinal layer, the layer closest to the basal lamina. Stem cells within the  
lining of the gut provide for a rapid renewal rate of this tissue. ESCs or keratinocytes  
obtained from the skin or lining of the gut of a patient or donor can be grown in tissue  
10 culture (Rheinwald, 1980, Meth. Cell Bio. 21A:229; Pittelkow and Scott, 1986, Mayo  
Clinic Proc. 61:771). If the ESCs are provided by a donor, a method for suppression of host  
versus graft reactivity (e.g., irradiation, drug or antibody administration to promote  
moderate immunosuppression) can also be used.

15 With respect to hematopoietic stem cells (HSC), any technique which provides for  
the isolation, propagation, and maintenance *in vitro* of HSC can be used in this embodiment  
of the invention. Techniques by which this may be accomplished include (a) the isolation  
and establishment of HSC cultures from bone marrow cells isolated from the future host, or  
a donor, or (b) the use of previously established long-term HSC cultures, which may be  
20 allogeneic or xenogeneic. Non-autologous HSC are used preferably in conjunction with a  
method of suppressing transplantation immune reactions of the future host/patient. In a  
particular embodiment of the present invention, human bone marrow cells can be obtained  
from the posterior iliac crest by needle aspiration (see e.g., Kodo et al., 1984, J. Clin. Invest.  
73:1377-1384). In a preferred embodiment of the present invention, the HSCs can be made  
25 highly enriched or in substantially pure form. This enrichment can be accomplished before,  
during, or after long-term culturing, and can be done by any techniques known in the art.  
Long-term cultures of bone marrow cells can be established and maintained by using, for  
example, modified Dexter cell culture techniques (Dexter et al., 1977, J. Cell Physiol.  
91:335) or Witlock-Witte culture techniques (Witlock and Witte, 1982, Proc. Natl. Acad.  
30 Sci. U.S.A. 79:3608-3612).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene  
therapy comprises an inducible promoter operably linked to the coding region, such that  
expression of the nucleic acid is controllable by controlling the presence or absence of the  
appropriate inducer of transcription.

35 Additional methods that can be adapted for use to deliver a nucleic acid encoding a  
RapR6 or functional derivative thereof are described below.

#### 5.4.6. METHODS OF REGULATING ACTIVITY OF RAPR6 PROTEIN AND/OR RAPR6 PATHWAYS

5

The activity of RapR6 protein can be regulated by modulating the interaction of RapR6 protein with its binding partners. In one embodiment, agents, e.g., antibodies, small organic or inorganic molecules, can be used to inhibit binding of a RapR6 binding partner such that rapamycin resistance and/or tumorigenesis is regulated. In another embodiment, 10 agents, e.g., antibodies, small organic or inorganic molecules, can be used to inhibit the activity of a protein in a RapR6 protein regulatory pathway, including but not limited to cyclin D1 or cdc2, such that rapamycin resistance and/or tumorigenesis is regulated.

#### 5.4.7. CANCER THERAPY BY TARGETING RAPR6 GENE, GENE PRODUCT, AND/OR OTHER RELATED CELLULAR MOLECULES

15

The methods and/or compositions described above for modulating RapR6 expression and/or activity may be used to treat patients who have a cancer as a result of defective regulation of a RapR6 gene. The methods and/or compositions may also be used in conjunction with rapamycin for treatment of a patient having a cancer which exhibits RapR6 mediated rapamycin resistance and/or tumorigenesis. Such therapies may be used to 20 treat cancers, including but not limited to, rhabdomyosarcoma, neuroblastoma and glioblastoma, small cell lung cancer, osteosarcoma, pancreatic cancer, breast and prostate cancer, murine melanoma and leukemia, and B-cell lymphoma.

In preferred embodiments, the methods and/or compositions of the invention are 25 used in conjunction with rapamycin for treatment of a patient having a cancer which exhibits RapR6 mediated rapamycin resistance and/or tumorigenesis. In such embodiments, the expression and/or activity of RapR6 are modulated to confer cancer cells sensitivity to rapamycin, thereby conferring or enhancing the efficacy of rapamycin therapy.

In a combination therapy, one or more compositions of the present invention can be 30 administered before, at the same time of, or after the administration of rapamycin. In one embodiment, the compositions of the invention are administered before the administration rapamycin. The time intervals between the administration of the compositions of the invention and rapamycin can be determined by routine experiments that are familiar to one 35 skilled person in the art. In one embodiment, rapamycin is given after the RapR6 protein level reaches a desirable threshold. The level of RapR6 protein can be determined by using any techniques described *supra*.

In another embodiment, the compositions of the invention are administered at the  
5 same time with rapamycin.

In still another embodiment, one or more of the compositions of the invention are  
also administered after the administration of rapamycin. Such administration can be  
beneficial especially when rapamycin has a longer half life than that of the one or more of  
10 the compositions of the invention used in the treatment.

It will be apparent to one skilled person in the art that any combination of different  
timing of the administration of the compositions of the invention and rapamycin can be  
used. For example, when rapamycin has a longer half life than that of the composition of  
the invention, it is preferable to administer the compositions of the invention before and  
15 after the administration of the rapamycin.

The frequency or intervals of administration of the compositions of the invention  
depends on the desired RapR6 level, which can be determined by any of the techniques  
described *supra*. The administration frequency of the compositions of the invention can be  
20 increased or decreased when the RapR6 protein level changes either higher or lower from  
the desired level.

The effects or benefits of administration of the compositions of the invention alone  
or in conjunction with rapamycin can be evaluated by any methods known in the art, e.g.,  
by methods that are based on measuring the survival rate, side effects, dosage requirement  
25 of rapamycin, or any combinations thereof. If the administration of the compositions of the  
invention achieves any one or more of the benefits in a patient, such as increasing the  
survival rate, decreasing side effects, lowering the dosage requirement for rapamycin, the  
compositions of the invention are said to have augmented the rapamycin therapy, and the  
method is said to have efficacy.  
30

#### 5.5. PHARMACEUTICAL FORMULATIONS AND ROUTES OF ADMINISTRATION

The compounds that are determined to affect RapR6 gene expression or gene  
product activity can be administered to a patient at therapeutically effective doses to treat or  
ameliorate disorders related to defective regulation of RapR6. A therapeutically effective  
35 dose refers to that amount of the compound sufficient to result in amelioration of rapamycin  
resistance and/or inhibition of the growth of cancer cells.

### 5.5.1. EFFECTIVE DOSE

5 Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Compounds which  
10 exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

15 The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the  
20 invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC<sub>50</sub> (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in  
25 plasma may be measured, for example, by high performance liquid chromatography.

### 5.5.2. FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present invention may  
30 be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

35 For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically



acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch,  
5 polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose,  
microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium  
stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or  
wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well  
known in the art. Liquid preparations for oral administration may take the form of, for  
10 example, solutions, syrups or suspensions, or they may be presented as a dry product for  
constitution with water or other suitable vehicle before use. Such liquid preparations may  
be prepared by conventional means with pharmaceutically acceptable additives such as  
suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats);  
emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily  
15 esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or  
propyl-*p*-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts,  
flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled  
20 release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges  
formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present  
25 invention are conveniently delivered in the form of an aerosol spray presentation from  
pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*,  
dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide  
or other suitable gas. In the case of a pressurized aerosol the dosage unit may be  
determined by providing a valve to deliver a metered amount. Capsules and cartridges of  
30 *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix  
of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*,  
by bolus injection or continuous infusion. Formulations for injection may be presented in  
unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative.  
35 The compositions may take such forms as suspensions, solutions or emulsions in oily or  
aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing

and/or dispersing agents. Alternatively, the active ingredient may be in powder form for  
5 constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

10 In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange  
15 resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser  
20 device may be accompanied by instructions for administration.

### 5.5.3. ROUTES OF ADMINISTRATION

Suitable routes of administration may, for example, include oral, rectal, transmucosal, transdermal, or intestinal administration; parenteral delivery, including  
25 intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into an affected area, often in a  
30 depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with an antibody specific for affected cells. The liposomes will be targeted to and taken up selectively by the cells.

### 35 5.5.4. PACKAGING

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may

for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser  
5 device may be accompanied by instructions for administration. Compositions comprising a  
compound of the invention formulated in a compatible pharmaceutical carrier may also be  
prepared, placed in an appropriate container, and labelled for treatment of an indicated  
condition. Suitable conditions indicated on the label may include treatment of a disease  
such as one characterized by insufficient, aberrant, or excessive RapR6 activity.

10

## 6. EXAMPLE

A mouse neuroblastoma cell line N2a cells (ATCC) were used for treatment with  
rapamycin. N2a cells are sensitive to rapamycin mediated cell growth inhibition at 10 nM  
(See, FIG. 7, MTT assay, a proliferation assay) and were used as target cells for the random  
15 homozygous knockout (RHKO) procedure to identify the genes and genetic pathways  
whose inactivation result in N2a cells resistant to rapamycin mediated cell growth  
inhibition.

N2a cells were transfected with a vector carrying a transactivator (FIG. 9D). The  
20 vector was modified from the expression regulator vector of the Tet-off™ system  
(Clontech). Several cell clones were generated. One of these clones (clone 44) shown  
strong transactivator activity was used as target cells for the infection of a retroviral vector  
(FIG. 9C) carrying a gene search construct as depicted in FIGS. 9A and 9C. The infected  
N2a cells were selected with puromycin within 4 days for cells undergone RHKO. Since  
25 the expression of puromycin is dependent on transactivator and can be suppressed by  
addition of Doxycycline in the culture medium, selection of RHKO clones was carried out in  
culture medium without Doxycycline. One week later, a RHKO library consists of more  
than 100,000 puromycin resistant cells was generated. The cells were treated with 1  $\mu$ M of  
Rapamycin (at this concentration all N2a cells were either killed or growth inhibited by  
30 rapamycin). Fourteen days later, 8 rapamycin resistant colonies were isolated and expanded  
into cell lines.

To confirm RHKO-dependent rapamycin resistance of the cell lines, cells were  
assayed for their resistance to rapamycin in the present and in the absence of doxycycline.  
RHKO dependent rapamycin resistance should be reverted to rapamycin sensitive in the  
35 presence of doxycycline. Clone 6 (RapR6 clone, see FIGS. 8A-8B) showed reversibility in  
the presence of doxycycline, indicating that random homozygous knockout of specific  
genes in the three clones resulted in the cellular resistant to rapamycin mediated growth

inhibition. FIGS. 8A-8B illustrate reversible inhibition by rapamycin in RapR6 clone (MTT proliferation assay). 8A: Solid bar, measurement when the knockout construct is expressed; shaded bar, measurement when the expression of the knockout construct is suppressed; and open bar, control. 8B: Calculated reversibility R according to Equation 1. FIG. 8C illustrates a RapR6 colony after 12 days of infection with an RHKO gene search vector.

FIGS. 8D-8E illustrate effects of expression of RapR6 gene on markers of G1 arrest in RapR6 clone. FIGS. 8D-8E show that inactivation of RapR6 gene increases the cellular level of Cyclin D1 (FIG. 8D) and cdc2 (FIG. 8E) but does not affect the cellular level of p70S6 (FIG. 8D). CDC2 protein kinase (also termed p34 protein kinase) which is activated by forming a complex with cyclin B and is required for the G2/M transition, i.e., the transition from G2 phase to mitosis, of the cell cycle. Reduction in CDC2 expression and/or activity has been shown to lead to G2/M arrest. These results indicate that RapR6 gene plays a role in cell tumorigenesis.

Southern blotting analysis of the RapR6 clone showed that the clone contains a single copy integration of the gene search vector, indicating only one gene has been inactivated by RHKO in each clone. The DNA sequences of the gene search vector were used to clone the genomic DNA fragment flanking the integrated gene search construct. HindIII was used to digest the genomic DNA. A 4 kb plasmid carrying a 1.5 kb genomic DNA flanking the RHKO insertion site was obtained. Three plasmids (designated as RapR61, RapR62, and RapR63, respectively) were isolated and sequenced using an oligo primer from gene search vector. FIG. 1A depicts a nucleotide sequence (SEQ ID NO:1) obtained from sequencing one of the plasmid. FIG. 1B depicts an alignment of the sequences obtained from the three plasmids. DNA sequences of the flanking genomic DNA fragments were obtained and used to search databases to identify the genes. FIG. 5D depicts the genomic location of the RHKO insertion site. The RHKO vector is inserted in the intron between exon 1 and exon 2 of the murine RapR6 gene. FIG. 2A depicts the nucleotide sequences of the transcript cDNA (SEQ ID NO:2) and of the murine exons 1-6 (SEQ ID NOS:4-9).

## 7. REFERENCES CITED

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent

application was specifically and individually indicated to be incorporated by reference in its  
5 entirety for all purposes.

Many modifications and variations of this invention can be made without departing  
from its spirit and scope, as will be apparent to those skilled in the art. The specific  
embodiments described herein are offered by way of example only, and the invention is to  
10 be limited only by the terms of the appended claims along with the full scope of equivalents  
to which such claims are entitled.

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